UNIVERSIDAD COMPLUTENSE DE MADRID

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TESIS DOCTORAL

Indicators of individual quality in the blue tit (*Cyanistes caeruleus*): parasitism, colour, paternity and aeging

Indicadores de calidad en el herrerillo común (*Cyanistes caeruleus*): parasitismo, color, paternidad y envejecimiento

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Elisa Pérez Badás

Directores

Santiago Merino Rodríguez
Francisco Javier Martínez González

Madrid, 2017

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Tesis doctoral 2017

Universidad Complutense de Madrid
Facultad de Ciencias Biológicas

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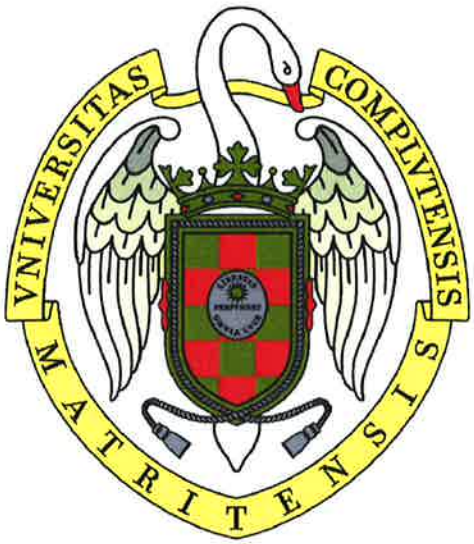

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Indicadores de calidad en el herrerillo común (*Cyanistes caeruleus*): parasitismo, color, paternidad y envejecimiento

Memoria presentada para optar al grado de Doctor por

Elisa Pérez Badás

Bajo la dirección de los Doctores

Santiago Merino Rodríguez y Fco. Javier Martínez González

Madrid – Marzo 2017

Santiago Merino Rodríguez, Científico Titular del Museo Nacional de Ciencias Naturales – CSIC

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THESIS STRUCTURE

This thesis is structured in eight main sections. The first section is a general INTRODUCTION, where the conceptual framework, aims and objectives of this work are described. The second section details the general METHODS that have been used throughout several chapters that constitute the present Thesis including the study species, study area and general laboratory procedures. The methodology is supplemented by the specific procedures used to develop each objective, which have been included in each relevant chapter. The following five sections include the chapters in standard scientific article format. Finally, an INTEGRATIVE DISCUSSION and CONCLUDING REMARKS are presented, which finalize the present PhD thesis.

'Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.'

—Maria Salomea Skłodowska-Curie

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ABSTRACT

The study of individual quality is paramount in evolutionary ecology if one is to unravel life-history trade-offs. The investment in self-maintenance, reproduction or survival may be determined by an individual's quality. Moreover, obtaining information from conspecifics might be essential in mate choice because mating with higher quality individuals could ensure direct or indirect benefits for the offspring.

Several studies have provided conclusive evidence that individuals can assess variation in the quality of conspecifics through several indicators of quality. In birds, plumage ornamentation has received much attention in the literature as an indicator of quality, and it is well known that females often prefer more conspicuously ornamented males. However, multiple ornaments may convey different pieces of information regarding quality, but no consistent pattern has emerged to this respect. Other indicators of quality may refer to physiological parameters that convey information about health status (for example, blood parasitic infections), or telomere shortening as a biomarker for ageing processes.

On this basis, the overall aim of this Thesis is to further increase the knowledge on indicators of individual quality in birds, using the blue tit (*Cyanistes caeruleus*) as model species. Data for correlational and experimental studies was collected in a blue tit population breeding in nest-boxes in central Spain (Valsaín, Segovia) during the 2012-2014 breeding seasons. Throughout several chapters in this Thesis we explored how infections by several parasite species, plumage colouration in multiple ornaments, mating strategies or ageing may relate to individual quality in adult and developing blue tits.

Using models based on avian colour vision we found that structural colouration in several ornaments may depend on stress during reproduction or development. During reproduction, intense parasitic infections by avian malaria-like

parasites had a differential effect on the quality of the white cheek feathers developed during the post-reproductive moult. Similarly, nestling blue tits that suffered from parasitic infections at the nest developed more saturated green tails and duller blue crowns after the post-juvenile moult. Moreover, we offer correlational evidence for assortative mating in the blue tit. Lower quality males paired with females that laid more pigmented eggs, but previous studies indicated that these females were in poor condition. Thus, male quality may be determinant in eggshell pigmentation, because poor quality male blue tits may provide less food to laying females during courtship, or, alternatively, the pair may breed in poor territories with less access to nutrients for females. Additionally, these males were younger and more likely to father extra-pair offspring, probably as a result of mating with poor quality females. In a different breeding season we showed that older and higher quality males were more ornamented and sired more extra-pair offspring, while bearing the risks of being infected with more blood parasites as a result of engaging in extra-pair copulations. Finally, our results suggest that the costs of reproduction may be mitigated in individuals in better nutritional status. After experimentally supplementing adult blue tits with antioxidants during the costly reproductive event, we observed reduced telomere shortening one year after.

To sum up, our findings support the idea that immunologically naïve individuals may suffer the costs from parasitic infections as reduced colour expression in structural ornaments, reduced paternity and accelerated ageing; but high quality individuals may overcome these costs while investing in ornamentation and maximizing reproductive success. Thus, this Thesis confirms that the implication of parasitic infections, structural colouration, mating strategies and nutritional status on reproductive performance, self-maintenance and senescence should not be overlooked.

Resumen

El estudio de la calidad individual tiene una enorme importancia en ecología evolutiva para desentrañar compromisos vitales. La calidad individual podría determinar la inversión destinada al mantenimiento del propio individuo, reproducción o supervivencia. Además, obtener información sobre otros miembros de la especie es esencial durante el emparejamiento, ya que formar pareja con individuos de alta calidad aseguraría beneficios de origen directo o indirecto para la descendencia.

Numerosos estudios han confirmado que los individuos son capaces de percibir variaciones en la calidad individual de sus congéneres a través de varios indicadores de calidad. En aves, la ornamentación del plumaje ha sido ampliamente estudiada como señal de calidad, y así, es bien sabido que las hembras prefieren emparejarse con machos más ornamentados. No obstante, múltiples ornamentos en un mismo individuo, podrían contener información distinta en cuanto a calidad, pero aún no se han descrito patrones consistentes a este respecto. Otros indicadores de calidad podrían ser parámetros fisiológicos que confieren información sobre el estado de salud (como por ejemplo, infecciones por parásitos sanguíneos), o el acortamiento de telómeros como marcador biológico en procesos de envejecimiento.

Sobre esta base, el objetivo principal de esta Tesis es el de profundizar en el conocimiento de los indicadores de calidad en el herrerillo común (*Cyanistes caeruleus*) como especie modelo. Los datos para los estudios correlacionales y experimentales que forman parte de esta tesis fueron recogidos en una población de herrerillo común que cría en cajas nido en el centro de España (Valsaín, Segovia), durante las primaveras de 2012-2014. A lo largo de los varios capítulos de esta Tesis exploramos cómo las infecciones por parte de varias especies de parásitos, la coloración en el plumaje en múltiples ornamentos, las estrategias de emparejamiento o el envejecimiento podrían relacionarse con la calidad individual en herrerillos adultos y polluelos.

Usando modelos basados en visión aviar encontramos que la coloración estructural de varios ornamentos podría depender del estrés durante reproducción o desarrollo. En la reproducción, las infecciones intensas por parásitos sanguíneos causantes de la malaria aviar tuvieron un efecto diferencial en la calidad del plumaje blanco de la mejilla desarrollado durante la muda post-reproductora. De forma similar, los polluelos de herrerillo que sufrieron infecciones por parásitos en el nido desarrollaron un plumaje más saturado en la cola y un azul más apagado en las plumas de la corona. Además, también descubrimos que los herrerillos en esta población parecen emparejarse atendiendo a su calidad. Los machos de peor calidad se emparejaron con hembras que pusieron huevos más pigmentados, lo que indicaría que dichas hembras también eran de peor calidad. Por tanto, la calidad del macho parece ser determinante en la pigmentación del huevo, quizá porque los machos de peor calidad alimentan menos a las hembras durante el cortejo, o, de forma alternativa, porque la pareja podría construir el nido en territorios de peor calidad con menor acceso a nutrientes para las hembras. Por otro lado, encontramos que estos machos eran más jóvenes y tenían más polluelos extra-pareja, probablemente como consecuencia de la menor calidad de su pareja. En otra estación reproductora encontramos que los machos más viejos y de mayor calidad eran los más ornamentados y los que tenían más polluelos extra-pareja. También fueron capaces de soportar un mayor riesgo de infección por parásitos como resultado de encuentros más frecuentes con individuos infectados durante las cópulas. Finalmente nuestros resultados sufieren que los costes de la reproducción podrían estar mitigados en individuos en un mejor estado nutricional. Tras la suplementación con antioxidantes durante una actividad costosa, como es la reproducción, observamos que el acortamiento de telómeros un año después del tratamiento era menor.

En resumen, nuestros resultados apoyan la idea de que los individuos inmunológicamente deprimidos podrían sufrir los costes de la infección por parásitos en forma de una menor expresión del color, menor paternidad y envejecimiento acelerado;

pero los individuos de mayor calidad serían capaces de asimilar estos costes y al mismo tiempo invertir en ornamentación y maximizar su éxito reproductivo. Por todo ello, esta tesis confirma que las implicaciones de las infecciones parasitarias, la coloración estructural, las estrategias de emparejamiento y el estado nutricional podrían tener en la actividad reproductora, el mantenimiento del propio individuo y la senescencia, no deberían ser ignoradas.

INTRODUCTION

Life-history theory, proposed in the 1950s, developed an analytical framework in which evolutionary ecologists could explore the trade-offs between investment in reproduction, growth and survival (Stearns 1992). An individual's quality may refer to the ability to maximize reproduction without compromising growth, self-maintenance or survival. Indeed, quality becomes especially relevant in the search for prospective mates. Because reproduction is costly, the sharing of reproductive activities with vigorous individuals or individuals that are in good health, may confer an advantage in life-history trade-offs for both members of the pair (Kokko et al. 2006). The benefits of mating with high-quality individuals arise from the high-quality genes, and subsequent fitness increase, that are passed on to progeny, as predicted by the Fisher-Zahavi process (Fisher 1915; Zahavi 1975). For all these reasons, the study of indicators of individual quality is of central importance to evolutionary biology, if one is to determine the mechanisms underpinning the evolution of mate choice and sexual conflict (Box 1), and ultimately, life-history decisions during reproduction.

In the following chapters, we will explore how reproductive success may be influenced by parasitic infections, extra-pair paternity behaviour or ageing patterns in birds. These parameters have commonly been used as biomarkers for individual quality in evolutionary ecology (Westneat and Stewart 2003a; Kotrschal et al. 2007; Sorci 2013), and they may be interrelated. Under the premise that high-quality individuals maximize reproduction while maintaining good general state, we will investigate patterns in bird behaviour during reproduction, and other mechanisms that may underlie the costs of bird reproduction. It is important to take into account that parasitic infections, paternity and ageing act as indicators of quality by providing information to the researcher, but with no function in animal communication (Morales 2006). However, there are additional indicators of individual quality that can be used as a cue to conspecifics, for example in

BOX 1. Sexual selection and individual quality: who chooses and who competes?

Darwin (1871) suggested the mechanism of **sexual selection** to explain the inheritance of characters that are limited to one sex "Sexual selection depends, not on a struggle for existence, but on a struggle between the males for possession of the females; the result is not death to the unsuccessful competitor, but few or no offspring", but he did not explain the underlying mechanisms explaining why females may choose males with more exaggerated traits (or secondary sexual characters). Among others (see Fisher 1930), an explanation for this is the **'handicap principle'** proposed by (Zahavi 1975), which regards the burden imposed by extravagance as a handicap that tests the quality of the bearer. Following this, many studies have experimentally demonstrated that females prefer to mate with more ornamented males (for example, regarding avian colouration displays, reviewed in Hill 2006).

The explanation behind female **mate choice** is that sexual selection arises from the evolution of anisogamy (difference in the size of gametes between males and females, and on the resources destined to them), and as a result of this, individuals of the same or different sex engage in interactions to gain access to gametes or mates (Andersson 1994). Four main models have been normally used in the literature to understand the evolution of mate choice (reviewed in Kokko et al. 2003): (i) **direct benefits**, which refer to the gain of fecundity, greater parental care, avoidance of infectious diseases, etc. that result from mating with vigorous males, and relate to increased fitness for the choosing individual; (ii) **indirect benefits**, on the contrary arise from genetic benefits that may be passed on to the succeeding generation, so that the offspring are more likely to reproduce; (iii) **sensory drive**, by which males evolved to exploit a pre-existing sensory bias, and thus the benefits for females rely on the production of more attractive sons (in line with the direct benefits model); and (iv) **sexually antagonistic coevolution**, or 'chase-away' model by which evolution is expected to strengthen female resistance to superfluous maladaptive matings, while males should coevolve 'seductive' traits to overcome female resistance. Other authors propose slightly different classifications for the mechanisms explaining the evolution of mating preferences (see Kirkpatrick and Ryan 1991).

Although research has mostly focused on competitive males and females as 'the choosy sex', males can also be choosy (reviewed in Edward and Chapman 2011). Thus, competing for mates and being choosy might not be mutually exclusive alternatives (Kokko et al. 2006). Male mate choice may be more likely to favour female traits that confer direct benefits (i.e. high fecundity). Still, when benefits from mating with high quality females arise, male mate choice is ensured.

mating decisions; and thus, they are also important for the above mentioned life-history trade-offs. Chemical, visual or sound signals are regarded as honest signals because they serve a communication purpose (the individual bearing the most ornamented trait is of better quality); and they are related to physiological parameters and other indicators of quality, as proposed by the signalling theory (Endler 1992; Schluter and Price 1993).

Therefore, in this thesis, we will also explore bird colouration as an indicator of individual quality. Birds provide an excellent model to investigate these premises (see the Study Species section in the **General Methods Chapter**).

PARASITIC INFECTIONS

'Stay away from lazy parasites,
who perch on you just to satisfy their needs,
they do not come to alleviate your burdens,
hence, their mission is to distract, detract and extract,
and make you live in abject poverty.'

—Michael B. Johnson
Poem, 2013

Infectious diseases pose a major threat to humans and other vertebrate hosts (Box 2). Under the theory of parasite evolution we expect increased parasite growth to cause negative effects on the host's fitness, commonly known as virulence (Ewald 1994). In particular, vector-borne infectious diseases are especially prone to increased virulence. For example, the parasite *Plasmodium falciparum* causes more deaths and illness in humans than any other vector-borne parasite (Luke and Hoffman 2003). However, other parasites from the same genera (i.e. *P. ovale*, *P. knowlesi*) are less virulent (Ewald 1994). Differing patterns of virulence are also found in the well-studied avian-malaria system. Avian blood parasites are widespread across all continents with the exception of the Antarctica, where vectors are absent (Atkinson and van Riper 1991; Valkiūnas 2005). The most common genera are *Haemoproteus*, *Plasmodium*, *Leucocytozoon*, *Hepatozoon*, *Trypanosoma* and nematode larvae (microfilariae). Among these, species of *Haemoproteus*, and other haemosporidians, mainly *Leucocytozoon*, are commonly referred to as avian malaria-like parasites (Pérez-Tris et al. 2005; Valkiūnas 2005). These genera have been found to commonly infect passerine birds, with varying prevalence across host species

(Loiseau et al. 2012), geographical range (Pérez-Tris et al. 2005; Merino et al. 2008; Szöllösi et al. 2011), and time (Cosgrove et al. 2008).

Vector-borne parasites infect a plethora of ectoparasitic arthropods as biological vectors (Figure) and birds as vertebrate hosts (Peirce 1981), where they develop into different stages during their cycle (Figure). The period between initial infection and

BOX 2. Parasites and vectors.

The word parasite stems directly from the Greek 'parasitos': "one who lives at another's expense". The biological definition of parasite, however, refers to an organism that lives off another one by causing some kind of harm, negative effect, or even eventually death on the host it occupies (Poulin 1998). Parasites are ubiquitous in nature: every living being has experienced, at least once during its lifetime, an encounter with parasites, or has a parasite living inside or in it (Zimmer 2001).

Almost every animal taxon includes parasitic species: viruses, bacteria, protozoans, nematodes, annelids, arthropods, vertebrates, fungi and plants (see Merino 2013). Although this is not an exhaustive list, it gives an idea of how cosmopolitan these organisms are in their distribution.

Among these, protozoans like *Plasmodium falciparum*, cause more deaths and illness in humans than any other vector-borne parasite. Their avian malaria relatives are also widespread and have become subjects of extensive research since the 20th century, because they cause severe diseases in some domestic and wild birds (Valkiūnas 2005). The figure below shows some of the most common haemoparasites infecting birds from free-living populations around the world and their most probable insect vectors (see Lainson 1960; Tomás et al 2008; but see Martínez-de la Puente et al 2011).

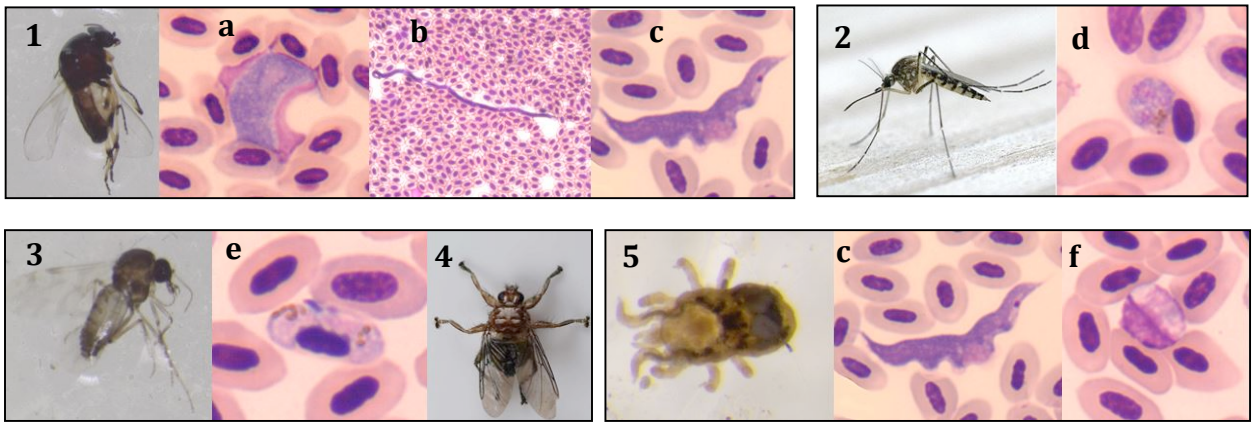

Figure 1. Most common vectors for each blood parasite species. 1) Biting midge (Diptera: Ceratopogonidae); 2) Mosquito (Diptera: Culicidae) (picture from pixshark.com); 3) Black fly (Diptera: Simuliidae); 4) Louse fly (Diptera: Hippoboscidae) (picture from studyblue.com); 5) Mite (Acari: *Dermanyssus gallinae*). a) *Leucocytozoon* spp.; b) Filarial nematode (*Microfilaria* spp.); c) *Trypanosoma* spp.; d) *Plasmodium* spp.; e) *Haemoproteus* spp.; f) *Lankesterella* spp.

release of gametocytes into the blood (the prepatency period) varies between parasite species: 12-13 days for haemoprotids (Fallis and Bennett 1961), 5-6 days for leucocytozoids (Desser and Bennett 1993), or as short as 1-2 days for trypanosomes (Bennett 1961) or 5 days for *Plasmodium relictum* (Valkiūnas 2005).

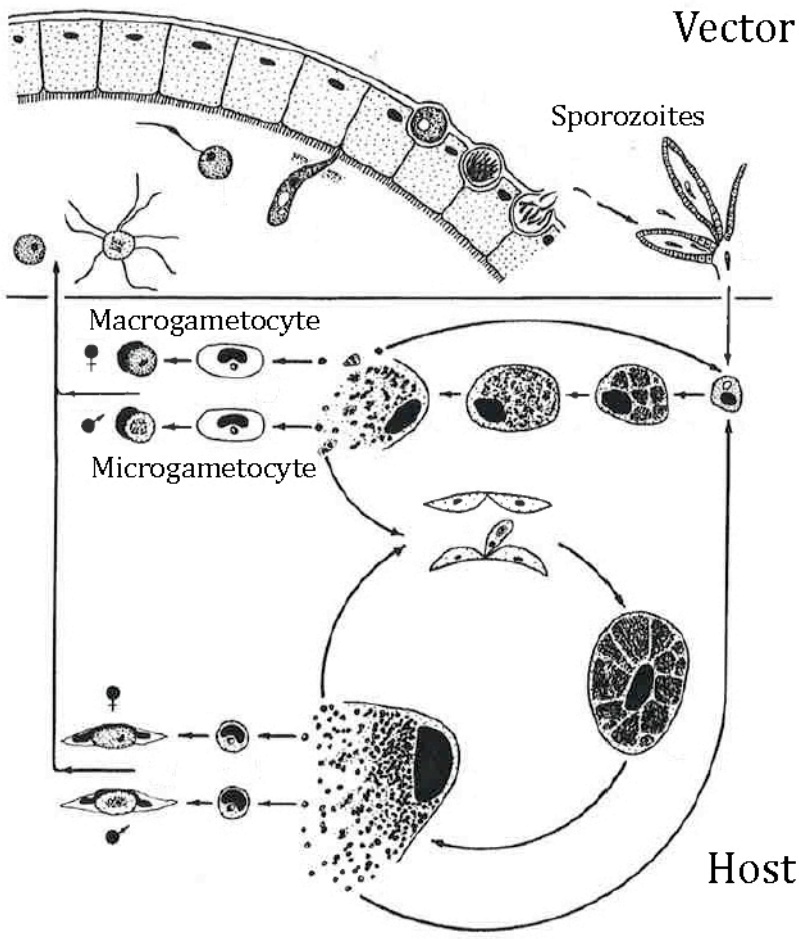

Figure 2. Life cycle of malarial and malaria-like parasites. The upper part takes place in the vector (sexual reproduction and release of sporozoites), and the lower part in the host (asexual reproduction until gametocytes develop in red blood cells)- modified from Valkiūnas (2005).

The costs of parasitism have been studied for long, but the way in which the host tolerates or resists the infection depends on many factors (Ewald 1994). Host and parasite genetics can shape the outcome of the infection through variation in the (i) intensity of

infection (parasitaemia), but other factors, such as (ii) environmental conditions, or (iii) the host nutritional status, can act by limiting or increasing its costs:

(i) **The intensity of parasitic infections** is usually determined by host and parasite genetics, and it can have profound repercussions in terms of host pathogenicity. However, it is also subjected to variation (Figure) if, for example, high quality individuals are able to mount stronger immune responses. At the acute phase of the infection, parasitaemia (number of parasites found in circulating blood stream) reaches its maximum in the avian host (Atkinson and van Riper 1991). High numbers of malarial parasites in blood are responsible for the destruction of erythrocytes by the host's immune system, leading to anaemia (Soni and Cox 1974). Pathological effects related to anaemia, including loss in body weight, have been reported in poultry animals, usually broiler chicken, ducks or turkeys infected with haemosporidian parasites (reviewed in Bennett et al. 1993). Likewise, the costs of the primary infection have been addressed in captive model birds after the experimental inoculation of parasites (Palinauskas et al. 2008; Zehtindjiev et al. 2008; Cellier-Holzem et al. 2010). In wild bird populations, malaria parasites are mainly studied during the chronic phase of the infection (Figure), when virulence and fitness costs are less obvious (Asghar et al. 2012). Chronic infections are usually associated to lighter infections and low number of parasites in circulating blood. Still, recent studies have demonstrated that defensive responses against malarial parasites may be an important fitness determinant in birds suffering from chronic infections (Davidar and Morton 1993; Marzal et al. 2008; Asghar et al. 2011; Lachish et al. 2011). A handful of medication experiments in the wild have successfully reported short and long-term costs of parasitic infections on different avian hosts (Merino et al. 2000; Marzal et al. 2005; Knowles et al. 2010; Martínez-de la Puente et al. 2010; Karell et al. 2011). Thus, the intensity of the parasitic infection might not always be so straightforward, as it strongly

depends on the relationship between the host's ability to fight the infection and parasite virulence (Vale et al. 2011).

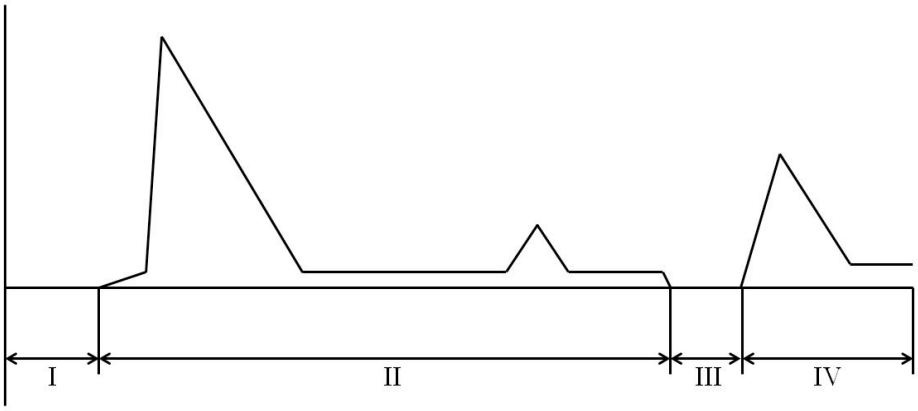

Figure 3. Dynamics of the infection by malarial parasites in birds. I- prepatent period (the parasite develops in internal organs); II- primary parasitaemia; III- latent stage of infection (the parasite is absent from circulating blood but persists in internal organs); IV- secondary parasitaemia due to relapse. - modified from Valkiūnas (2005).

Moreover, in order to deepen our knowledge on the relationships between host and parasites in the wild, more studies on multiple parasitic infections on the same hosts are needed (Davidar and Morton 2006; Martínez–de la Puente 2008; Marzal et al. 2008; Rooyen et al. 2013; Pollitt et al. 2015). In several chapters throughout this thesis, we explore the effects of the intensity of multiple parasitic infections on the host's individual quality through parameters such as body condition, haemoglobin levels in blood, general performance (breeding success) or ageing; both in adults **(Chapters 1, 3, 4 and 5)** and developing birds **(Chapter 2)**.

(ii) **Environmental conditions**, can promote the increase in parasite population growth by favouring parasite transmission or imposing challenges to the host's immune system (Vale et al. 2011). In Mediterranean regions, vector abundance is generally limited to spring and summer, when conditions are favourable for arthropod populations to thrive

(Martínez–de la Puente 2008; Garamszegi 2011; Martínez-de La Puente et al. 2013). But challenges in the host's immune system can also be determined by host dynamics. In fact, environmental conditions during spring make this the most appropriate season for bird reproduction, because it coincides with an increase in food availability essential for nestling provisioning. However, this is also a time when the negative effects of parasitic infections are especially patent. Adults, during their costly reproductive stage, and developing nestlings, can be more susceptible to newly acquired parasitic infections because they are immunodepressed (Dowell 2001). Alternatively, under chronic infections, the weakening of immunity during reproduction frequently leads to a short-term increase in the number of parasites in the blood (recrudescence or relapses of the infection, see below, Valkiūnas 2005).

Therefore, high-quality individuals who are able to cope with parasite infections during reproduction may counteract the costs of infections. Additionally, recent studies have confirmed that the conditions experienced during early-life can have a profound impact on future fitness and survival (Lindström 1999; Orledge et al. 2012; Costantini et al. 2012; Herborn et al. 2014), and in this respect, parasitic infections in developing nestlings may also play an important role in future reproductive success. These premises will be explored in **Chapters 1 and 2**.

(iii) A less explored topic is how **nutritional status** could confer benefits under the challenge of parasitic infections. Nutritional status is thought to play an important part on parasite resistance through activation of the immune system (Allen and Ullrey 2004). Several vitamins and micronutrients are known for their ability to improve immune functioning in birds (Soler et al. 2003; Brommer 2004; da Silva et al. 2011) or reduce oxidative stress (Giraudeau et al. 2013). However, few studies have reported the effects of food supplementation on the prevalence of infections or parasitaemia (Soler et al. 2003;

Cornet et al. 2014). To date, no study has compared the effects of supplementation and medication treatments on pre-existing parasitic infections in wild birds. We explore this in **Chapter 5**.

Indeed, parasites have an impact on health status and general performance in adult and developing birds. Individuals may use visual signals such as ornamental colouration to extract information about parasitic infections in conspecifics.

BIRD COLOURATION

'Why do two colours, put one next to the other, sing?
Can one really explain this? No.
Just as one can never learn how to paint.'

— Pablo Picasso

Since the Hamilton-Zuk hypothesis explained ornamental colouration as a means of communicating resistance to parasites (Hamilton and Zuk 1982), feather colouration displays have been the cornerstone of signalling theory in evolutionary ecology. In this context, females may choose more ornamented males, as these are high-quality mates that will produce disease resistant and ornamented sons (see Box 1). Within species, there is empirical evidence of the negative association between male ornamentation and parasite loads, and female preference for ornamented and parasite-free males (Milinski and Bakker 1990; Møller 1990; for a review see Hill 2006b).

However, there are two primary mechanisms that account for plumage colouration in birds and thus, their association with parasitic infections may differ. The first, **structural colouration**, is responsible for ultraviolet colouration, blues and greens, and it depends on the nanostructure of the feather (Prum 2006). It results from coherent

scattering when light is reflected by the melanosomes (granules of melanin), which are arranged between layers of keratin and air in the feather barbules (Doucet and Meadows 2009). The particular arrangement of these structures creates iridescence (or potential changes in colour with the angle of observation), a visual characteristic that is broadly distributed throughout class Aves. Therefore, structurally organised layers may be costly to produce (Dale 2006), and only high quality individuals may cope with parasitic infections while still showing highly organised feather structures. Indeed, some studies have provided correlational evidence of the relationship between structural plumage colouration and individual health in birds (Doucet and Montgomerie 2003b; Costa and Macedo 2005). However, few experimental studies confirm this: Hill et al. (2005) reported reduced iridescent coloration in wild turkeys (*Meleagris gallopavo*) after an experimental infection with coccidial parasites; and Shawkey et al. (2007) showed that bacterial infections can alter structural colouration by degrading the spongy layer of feather barbs in male eastern bluebirds (*Sialia sialis*). Different types of structural non-iridescent ornaments and its association with individual health have also remained understudied: for example, green colouration, combines yellow pigments and a structural component (Prum 2006), but no studies have explored the link between parasitic infections and deposition of colour in these plumage patches.

A particular type of structural colouration in which pigments are absent is achromatic colouration (Doucet et al. 2005; Shawkey and Hill 2005; Galván 2010). White ornaments are produced by incoherent scattering of all visible light wavelengths (in contrast to structural iridescent colouration, which is produced by coherent scattering) (Prum 2006). Brightness in achromatic white feathers has been related to dominance rank (Woodcock et al. 2005), female preference for more ornamented males (Mennill et al. 2003), resistance to oxidative stress (López-Arrabé et al. 2014), and body condition

(Galván 2010; D'Alba et al. 2011) in several passerines, but to date, a relationship between parasitic infections and white feather colouration has not been found.

The second mechanism of colour production is **pigment-based plumage colouration**. Two main pigments can be deposited in feathers (although see Box 3, Figure 4): melanins (responsible for black and brown colours), and carotenoids (responsible for yellows, reds and oranges). The physiological pathway to become more colourful in carotenoid-based ornaments involves incorporating these pigments from the diet (Hill et al. 2002), and an efficient metabolism, which is energetically challenging (McGraw 2006). Moreover, carotenoids are important immunomodulators and antioxidants (Chew 1993; Alonso-Alvarez et al. 2004). Thus, under the view that carotenoids are traded-off between immunity and ornamental colours (Von Schantz et al. 1999; Alonso-Alvarez et al. 2004), many studies have related carotenoid-based ornament to parasite loads (reviewed in Hill 2006b). Other studies, however, have failed to find such negative relationships (Seutin 1994; Fitze and Richner 2002).

BOX 3. Eggshell pigmentation by porphyrins

Uncommon pigments are responsible for bird colouration not in feathers, but in other colourful structures. One of these structures is the eggshell. The striking variety of eggshell colours and patterns has puzzled ecologists for long (Figure 4), and the function and evolution of eggshell colouration has been extensively studied. Several hypotheses have been suggested to explain why eggshells are pigmented: camouflage, avoiding brood parasitism, thermoregulation, UV-protection, signalling, or eggshell strengthening (reviewed in Cherry and Gosler 2010). However, experimental demonstration of some of these hypotheses is still lacking.

The main pigments involved in eggshell colouration are the tetrapyrrolic porphyrins, which are intimately related to the metabolism of haemoglobin (Ponka 1999). Among them, protoporphyrin confers brown spots to the eggshell, while biliverdin mainly appears in green eggshells. These pigments have been related to female quality because of their nature: biliverdin is a pro-oxidant while protoporphyrin is an antioxidant (Afonso et al 1999, Moreno and Osorno 2003).

The relationship between eggshell pigmentation and male quality, is however, less clear (but see Martínez-de la Puente et al 2007, Martínez-Padilla et al 2010).

Figure 4. a) Eggshell colouration and patterns among bird species (picture from animalpicturesociety.com) b) Eggshell pigmentation varies also within the same species, blue tit (*Cyanistes caeruleus*).

On the contrary, relating ornamental colouration to other aspects of individual quality seems more straightforward. A study by Kraaijeveld et al. (2007) shows a comprehensive list of species where correlations between ornaments and quality components have been found (i.e. several breeding parameters, Doucet and Montgomerie 2003a; immune function, Møller and Petrie 2002; nutritional stress, McGraw et al. 2002; body condition, Doucet 2002). All major types of ornaments have been related to various aspects of quality because they are thought to be costly to produce (even achromatic colouration, which could be regarded as the 'cheapest') (for a review, see Dale 2006).

Feather colour production occurs at moulting. Plumage colour is a dynamic trait, but contrary to other keratinized structures with continuous growth, it needs to be replaced as new colours are deposited onto newly formed feathers (Senar 2004) (Box 4). Birds change their feathers either before the breeding season (pre-nuptial moult) or

immediately after (post-nuptial moult), avoiding overlaps with migration or extreme winter conditions that could hamper the individual's nutritional status (Jenni and Winkler 1994). The reason behind this particular timing is that moult is an energetically demanding process, as the complete moult could be compared to replacing over 20% of the individual's weight (Senar 2004). Thus, it is likely that this process is affected by nutritional and health status (Sanz et al. 2004; Marzal et al. 2013; Trigo and Mota 2016), and so is pigment deposition on new feathers and structural organization (Hill and McGraw 2006).

BOX 4. The moult

Moulting in birds is not a continuous process, but instead the new feather appears by pushing the old one, which is lost for a certain period of time. The newly moulted feather will keep growing until its maximum length (see figure below). This lack of complete feather during a certain period of time explains why the moult is likely to hamper a bird's flight ability, thermoregulation, permeability, or communication through colour signalling (Senar 2004). In fact, replacing more than five feathers at a time has been shown to prevent birds from flying (Jenni and Winkler 1994).

Most bird species undergo a complete post-reproductive moult. Other species incur in an additional partial moult before the reproductive season in order to incorporate newly deposited colours to attract mates.

The moult is different in juveniles. Nestlings are equipped with less and softer feathers, and these are useful for thermoregulation at the nest but need to be replaced before the individual's first reproductive season (see figure below). Therefore, coinciding with the adults' moult process, juveniles also replace some body feathers in order to survive cold winters and allow for search mating in the following spring (Senar 2004).

From left to right: moulting adult male and female greenfinch (*Carduelis chloris*), individuals showing their juvenile, softer head feathers before the partial post-juvenile moult: European jay (*Garrulus glandarius*), goldfinch (*Carduelis carduelis*). Pictures from David Norman, Merseyside ringing group.

For all these reasons, in **Chapter 1** we explore the changes in feather colour before and after the moult and how this process could be related to indicators of quality during the reproductive season. In **Chapters 1 (adults) and 2 (nestlings),** we further explore the effects of multiple parasitic infections and other indicators of quality in several structural and carotenoid-based ornaments. Indeed, another noteworthy point on the study of bird colouration in relation to individual quality refers to the information that may be contained in multiple ornaments. The groundbreaking paper by Møller and Pomiankowski (1993) proposed three hypothesis to explain multiple plumage colouration on the basis of quality: (i) different ornaments may reflect different aspects of individual quality (multiple messages hypothesis), (ii) different ornaments may provide information on similar aspects of quality (redundant messages hypothesis), or (iii) ornaments do not reveal quality but they are maintained because they are relatively cheap to produce and females show certain preferences for more ornamentation. Little support has been given to the redundant messages (reviewed in Candolin 2003; but see Ferrer et al. 2015) or the unreliable messages hypotheses. In accordance with the multiple messages hypothesis, an increasing number of studies support that individuals may rely on several ornamental cues in order to select among suitable mates (McGraw et al. 2002; Doucet and Montgomerie 2003a; Silva et al. 2008; Dongen and Mulder 2009; Freeman-Gallant et al. 2010; Vergara and Fargallo 2011).

MATING STRATEGIES (EXTRA-PAIR PATERNITY)

'The numbers relevant to natural selection are
the number of genes passed on into the succeeding generation.'

—Paul W. Ewald
'Evolution of infectious disease', 1994

Indeed, selecting among suitable mates may be a daunting task. Life-history trade-offs predict that mating with poorer individuals may result in an imbalance between maximizing reproduction and self-maintenance (as explained above). Thus, why should individuals content themselves with one mate when there is a risk that the most suitable is elsewhere? An alternative to this conundrum is to engage in extra-pair matings, which may be more common than initially expected in socially monogamous bird species (Birkhead et al. 1987; Birkhead and Møller 1992).

Traditionally, extra-pair copulations have been related to high quality males that were able to increase reproductive success by mating with several females (Westneat et al. 1990). However, it is now well established that females may also play an active role in soliciting extra-pair copulations (Birkhead and Møller 1993). If males vary in their individual quality, an alternative to monogamy is that females seek extra-pair copulations with higher quality males (Petrie et al. 1998). Several hypotheses have been proposed in order to explain the benefits for extra-pair paternity to females: direct benefits of additional male provisioning, fertility insurance, good genes, genetic compatibility, or increased genetic diversity among offspring (reviewed in Charmantier et al. 2004). Still, few studies have collected enough data of this type to discriminate between the various hypotheses (but see Kempenaers et al. 1997; Krokene et al. 1998). In addition, these hypotheses are not mutually exclusive. For example females can seek additional matings

for ‘good genes’, but these may at the same time lead to fertility enhancement or even further ecological direct benefits (Charmantier et al. 2004).

Both direct and indirect benefits (or ‘good genes’) could arise from mating with more ornamented individuals out of the social pair. Several studies have related an increase in plumage ornamentation to the presence of extra-pair paternity in birds (Møller and Birkhead 1994; Sundberg and Dixon 1996; Delhey et al. 2003; Doucet et al. 2005; Helfenstein et al. 2008); but others have failed to find an association (Kappes et al. 2009), or experimental confirmation for correlational studies (Delhey et al. 2007). Additional matings with individuals in better health status may also confer an advantage. However, how parasitic infections relate to the likelihood of siring extra-pair young is understudied and inconclusive (Wagner Davidar, P., Schug, M.D., Morton, E.S. 1997; MacDougall-Shackleton et al. 2002; Podmokła et al. 2015). Thus, further studies on multiple indicators of quality and their association with extra-pair paternity are needed (Westneat and Stewart 2003b). The relationships between multiple colour signals, parasitic infections and other indicators of quality are explored in **Chapters 3 and 4**.

Yet, it is straightforward to understand that activities related to reproduction, such as mate searching, are costly and therefore, they might incur in long-term costs. Several studies have now shown that breeding success typically increases in the form of enhanced annual productivity or offspring survival with increasing age, followed by a reproductive maximum in the form of a single peak or plateau, and thereafter, a decline (Newton and Rothery 1997; Murphy 2007; Bouwhuis et al. 2009; Hammers et al. 2012; Potti et al. 2013). Indeed, in most free-living populations the decline in reproductive success with age is commonly known as senescence (Hamilton 1966).

AGEING

'The tragedy of old age is not that one is old, but that one is young.'

—Oscar Wilde

To explain senescence, three non-mutually exclusive hypotheses have been proposed. The accumulation of mutations hypothesis (Medawar 1952) states that senescence is due to deleterious mutations that accumulate over evolutionary time. The antagonistic pleiotropy hypothesis (Williams 1957) suggests that senescence can occur because natural selection favours genes that have a positive impact on individual performance in the early stages of life, but a negative impact in its later stages. A third mainstream theory of ageing, the disposable soma theory, suggests that the body must budget the amount of energy available towards reproduction at the expense of somatic repair (Kirkwood and Rose 1991). In any case, studies on senescence in wild bird populations would benefit from inside on how physiological and nutritional stress may affect ageing.

During reproduction, the costs induced by several activities (mating, weakened immune system, nestling provisioning, etc.) can increase oxidative stress, creating a disequilibrium between cell maintenance and antioxidant defences that is especially evident when parental effort exceeds what individuals are prepared to sustain (Christe et al. 2012; Blount et al. 2016; Alonso-Álvarez et al. 2017). These costs emphasize the importance of maintaining the balance between self-maintenance and reproduction in life-history decisions and the need to include physiological traits in ecological studies in order to understand the evolution of life histories (Wegmann et al. 2015).

Recently, much attention has been given to one of these physiological traits: telomere shortening, which is used as a biomarker for cellular senescence

(Blackburn 1991). Telomeres are short tandem repeats at the end of chromosomes in all eukaryotic cells, and they are known to shorten with each cell division and with oxidative stress (von Zglinicki 2002; Kotrschal et al. 2007). When telomeres reach a critical length, the degenerative process of ageing and cell apoptosis is triggered, which makes the rate of telomere degradation a suitable proxy for ageing (Bize et al. 2009; Boonekamp et al. 2014). In birds, experimental studies have shown the costs of reproduction on telomere dynamics by increasing parental effort (Reichert et al. 2014; Hau et al. 2015) or stress in development during early-life (i.e. increased brood size Nettle et al. 2013; Boonekamp et al. 2014; Nettle et al. 2015). Thus, longer telomeres and lower telomere attrition have been related to high quality individuals, but only indirectly (Bize et al. 2009; Salomons et al. 2009; Barrett et al. 2013). A direct approach would require that some individuals have instead ameliorated costs under the costly reproductive event, in order to attribute the observed differences in the rate of telomere shortening to an improvement in individual quality (i.e. by increasing nutritional status or decreasing parasitic infections). We explore these premises in **Chapter 5**.

The striking variety of life-history strategies seen in the animal world has fuelled the interest in indicators of quality in evolutionary ecology, as revealed by the multiple studies on physiological or signalling traits described above. However, studies that combine information offered by multiple traits in the same individual promise to be an interesting avenue for research if one is to disentangle some of the associations between life-history decisions and individual quality.

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OBJECTIVES

The overall aim of the present Thesis is to increase knowledge from an evolutionary perspective of the indicators of quality in cavity-nesting birds such as the Blue tit. This PhD Thesis includes descriptive studies and field experiments, which aim to explain the relationship between parasitism, colour, paternity and ageing.

The specific aims of the present thesis are listed below:

I. In **Chapter 1,** we aimed at investigating colour change in plumage patches that differ in their main mechanism of colour production (structural, pigmentary, or both), and related theses changes to parasitic infections during reproduction and to other breeding parameters. Because the carry-over effects of reproduction on moult and feather colouration may have an effect on mating patterns in the following reproductive event, the second aim of this chapter was to investigate the effects of colour change and breeding parameters on matting patterns in the consecutive breeding season.

II. In **Chapter 2,** we administered the following treatments aimed at reducing the parasite community infecting nestlings: a group of nests was sprayed with an insecticide, while, in other group, nestlings were administered with anti-malarial medication. We explored the effect of the treatment on feather colouration in two plumage patches in blue tit nestlings: the yellow breast and the blue-green tail. Additionally, we explored the effect of the treatment on feather colouration in the long-term, and we tested this by recapturing some individuals in winter.

III. In **Chapter 3**, we explored the relationship between eggshell pigmentation and individual quality of breeding blue tits. We used ornamentation in several plumage patches, blood parasite infections and extra-pair paternity as indicators of individual quality. Secondary aims of this chapter were to explore whether the female and her

partner's age predict eggshell pigmentation, and to test breeding phenology and reproductive trait effects on spotting coverage as a measure for eggshell pigmentation.

IV. In **Chapter 4**, we aimed at investigating which individual quality variables explain most of the variation in male paternity in the blue tit (ornamentation, parasitic infections or condition variables). In addition to this, we investigated discriminability of male colour change between seasons aimed at exploring whether polygynous males developed similar feather colour after the moult.

V. In **Chapter 5**, we investigated whether medication against blood parasites, supplementation or the combination of both (i) reflect improved fitness parameters in the short term and (ii) are efficient in reducing parasite loads. We then evaluated the effect of the treatments on fitness parameters and telomere shortening one year after administration of the treatment.

GENERAL METHODS

The following sections describe the general characteristics for the study species and study area, with particular remarks on the reasons that make the blue tit a perfect model organism to study indicators of individual quality during reproduction. We will also provide a brief description for some of the molecular methods and colour analyses that are common to several chapters. The specific methodological procedures are outlined in each chapter. We detected and/ or quantified blood parasites molecularly in **Chapters 1, 2, 3, 4, and 5**; in **Chapters 1, 2, 3 and 4** we used avian visual models in the study of animal colouration and detailed the procedure used in the field when measuring feather colouration; in **Chapters 2, 3** and **4** we detail the methodology for paternity analyses; and in **Chapter 5** we used telomere shortening analyses as a biological marker for ageing.

STUDY SPECIES

' Little bird of beauty,
God's treasure,
isn't it amazing how a blue tit
can give you so much pleasure?'

—Ben Franklin
Blue Tit poem, 2005

The Eurasian Blue Tit (*Cyanistes caeruleus*) (Linneo 1758) is a resident insectivorous passerine from the family Paridae, commonly found throughout the western Palearctic. Small (7-11 g), single-brooded, short-lived, and socially monogamous, the blue tit is a hole-nesting species that readily breeds in man-made nestboxes when provided (Perrins 1979). Individuals are found typically in deciduous forests, but can also be seen in evergreen and mixed forest habitats (Campbell and Fergurson-Lees 1972).

Adult blue tits are characteristically very colourful birds, which make this a suitable model organism in which to study ornamental colouration in relation to individual quality, mating patterns, and sexual selection. Adults typically have a white cheek and forehead, green back, yellow breast and blue crown, tail and wings (Fig. 1). The head pattern shows a black band from the beak to the back of the head, and another black band with variable width across the yellow breast plumage. Females are usually duller than males in the blue crown, wing coverts and band of the neck, but this difference is not always easy to see to the human eye. In fact, this species was not regarded as sexually dichromatic until recently, when it was shown that blue tits can perceive light from the ultraviolet (UV) region of the spectrum (Hunt et al. 1998), and differences between males and female UV reflectance were found mainly in the blue crown (Hunt et al. 1999; Sheldon et al. 1999). The juveniles' plumage colouration is manly green in their wings and back, yellow in their cheek and breast, and green-bluish tail. Blue tits undergo a complete post-breeding moult that is usually finished in early September, and this plumage colouration is maintained throughout the following winter and breeding season (Nilsson and Svensson 1996). This situation is ideal for studies on the carry-over effects of reproduction, for instance, on feather colouration (**Chapter 1**). The partial post-juvenile moult includes body feathers, the central pair of tail feathers, lesser and median coverts, all greater coverts and some tertials; some birds can moult some alula feathers and all tertials; and this is usually finished in October (Svensson 1992).

Figure 1. Blue tit (*Cyanistes caeruleus*). Picture from Ángel M. Sánchez.

The onset of the reproductive season varies with locality and year, but nest building usually takes place in March or April, occasionally extended into early May (Perrins 1979). Because of their abundance in woodlands, plant fibres containing cellulose are the most common materials used by blue tits to bind and provide good heat-insulation properties to the structure of the nest (Collias and Collias 1984). Following the typical three-layered design proposed by Hansell (2000), several strata can be recognised in a blue tit nest, which is built only by the female (Cramp and Perrins 1998). The initial nest foundation consists of a sturdy structural layer made of moss, and over this, an asymmetric cup, in which the female lays the eggs, is lined with fine dry grass, hair, wool or some feathers (Fig. 2). Although this is the basic nest structure of blue tits, the addition of several pieces of aromatic green plants has been recorded (Tomás et al. 2012). Blue tits nesting in artificial boxes usually start by filling the corners, and the lining is more likely to include a larger amount of feathers, grass and vegetable fibres.

Figure 2. Examples of blue tit nests breeding in nest boxes in our study population

Laying date typically starts around mid-April, clutch size ranging from 4 to 14 eggs (Gibb 1950). In the present study population, the mean clutch size between 1999 and 2012 was 9.17 eggs (Rivero-de Aguilar 2015). Females incubate the eggs for approximately 13 days, and both parents take part in nestling provisioning. Blue tit nestlings are mostly fed caterpillars found on young oak tree leaves (Bańbura et al. 1994) until they fledge, 17–20 days elapsed after the date of hatching. The mean number of fledglings in our study population is 7.8 (Fargallo 1997).

During the reproductive season, the individuals in this study population are commonly infected with several species of parasites. Furthermore, between-years adult recapture in our study area is high (i.e. 46% adult females and 58% males from season 2010 were recaptured in season 2011), making this blue tit population particularly suitable for longitudinal studies in which sampling of the same individuals is needed (**Chapters 1, 4 and 5**).

Finally, a noteworthy point is that two sub-species of blue tit can be found in the Iberian Peninsula: *Cyanistes caeruleus* ssp. *caeruleus*, distributed throughout Europe, and ssp. *oligastrae*, mainly occupying Spain and Southern France (Kvist et al. 2004). Thus, Mediterranean populations of blue tit are at the southern end of the distribution range of the species (Cramp and Perrins 1998). Particular climatic and habitat conditions representative of each study area may affect blue tit phenology (Amininasab et al. 2016).

STUDY AREA

'Eres tú, Guadarrama, Viejo amigo,
La sierra gris y blanca,
La sierra de mis tardes madrileñas,
Que yo veía en el azul pintada.'

—Antonio Machado
'Camino de Valsaín', 1911

The blue tit population under study is located in a deciduous forest in 'Montes de Valsaín' (Mata de Navalparaíso, 40º 53' 74N, 4º 01' O, 1200 m.a.s.l.), on the northern slope of the 'Sierra de Guadarrama' and under the legal protection of the Spanish National Park regime. The area is situated in the Segovia province and it covers a surface area of 10,700 hectares. The forest is mainly composed of young oak trees (*Quercus pyrenaica*) mixed with some pine trees (*Pinus silvestris*) and ash (*Fraxinus angustifolia*) (Fig. 3). The use of nestboxes in this study area has increased the breeding population of insectivorous passerine species, because natural cavities are less abundant than in forests composed of mature oak trees (Sanz 2000). The understory vegetation typically consists of laurel-leaved rockrose (*Cistus laurifolius*), cane-fruit species (*Rubus* sp.) and other flowering shrub species (*Rosa* sp.). Climatic conditions in the area are those typical of the supra-mediterranean floor in subhumid ombroclimates, with mean annual temperatures between 8-12ºC (Izco 1984). However, there is substantial interannual variation during spring, and extreme episodes with longer-retained snow cover can occasionally affect the blue tit's breeding biology (Glądalski et al. 2014).

Over 300 human-made wooden nestboxes were placed in the forest in 1991, and they have been monitored yearly ever since (Fargallo 1997). Nestboxes were hung from branches at about 4 m above the ground, thus, avoiding potential height differences (for

example, in avian exposure to insect attacks Fallis and Smith 1964); at locations of approximately 25 m apart (for dimensions and structure of nestboxes see the appendix in Lambrechts et al. 2010). All nestboxes were emptied prior to bird occupation and, during the breeding season, they were inspected periodically to determine the reproductive stages of the birds. Other passerine species that may occupy nestboxes in our study population are the pied-flycatcher (*Ficedula hipoleuca*), and less frequently, the great tit (*Parus major*) and the nuthatch (*Sitta europaea*).

Fieldwork took place during the breeding seasons of 2012 (**Chapters 3 and 5**), 2013 (**Chapters 1, 2, 4 and 5**) and 2014 (**Chapters 1 and 4**), although data on the exact age of adult individuals was extracted from previous ringing records from the present study population.

Figure 3. Study area in early (left) and late spring (right).

PARASITE DETECTION AND QUANTIFICATION

'A marriage of molecular and microscopic approaches
is already an established practice
in studies of haemosporidians in their vertebrate hosts.'

—Gediminas Valkiūnas
Molecular Ecology, 2011

Primers specificity and qPCR validation for *Haemoproteus majoris*

Primers were designed on the basis of DNA sequences that were previously obtained for each parasite (Table 1). The lower genetic identity exhibited between the haplotype leuB and leuA/leuA1 allowed the design of a specific pair of primers to detect the haplotype leuB, but not to differentiate leuA from leuA1 (which shared a genetic identity of 99.5%). Thus, the variable *Leucocytozoon* A includes haplotypes A and A1. The specificity of all pairs of primers was checked using a mix sample containing DNA from all parasites. In addition to the melting curve data, the amplicons obtained with each pair of primers were sequenced to corroborate specificity.

The qPCR was highly efficient: it only failed to detect one event of *Haemoproteus* infection. Comparison of qPCR vs. microscopic analyses was done for 89 females and 102 males. The correlation between parasitaemia as obtained from qPCR and from blood smears was highly significant ($r=0.81$, $t=19.03$, $df=189$, $P<0.0001$).

Haemoproteus majoris parasites were also detected and quantified from blood smears under the microscope at high magnification of (x 1,000). The same parasitologist (E.P.B.) examined all blood samples using an Olympus BX41 light microscope equipped with an Olympus SC30 digital camera. In order to estimate the density of parasites, we counted the number of infected cells per 2,000 erythrocytes in at least 40-50 fields

(Merino and Potti 1995; Merino et al. 1997). Additionally, we counted the number of juvenile vs. mature stages of the parasite in blood (Fig.4). Parasites whose length is usually less than the length of the nucleus of the infected erythrocyte are referred to as 'young gametocytes' (Valkiūnas 2005).

The detection and quantification of nest ectoparasites is detailed in **Chapter 2**.

Table 1. Primer sequences and PCR conditions for parasite detection and quantification.

Primers	Sequence 5'→ 3'	Gene (size)*	Annealing†	Extension‡	Species (specificity)§
qHaeF	GACTTGTTTCATGGATTTGTGGA	*Cyt b* (149)	60°C - 30	30	*Haemoproteus majoris* Cyan2 (HQ384262)
qHaeR	AGGATTAGAGCTACCTTGTAAG				
qPlasF	ATCTTGTAAGTGACCCAACC	*Cyt b* (139)	60°C - 30	30	*Plasmodium* sp. Cyan1 (FJ494966)
qPlasR	GCTGTATCATACCCTAAAGGATTTG				
qLeuF1	ACCTTTATCATGGTATAGTGGT	*Cyt b* (166)	58°C - 30	30	*Leucocytozoon* sp. leuA (KJ415278) and leuA1 (KJ415279)
qLeuAR	AAATCCACCACATACCCAG				
qLeuF3	AGTTTCTGGGGAGCAACTG	*Cyt b* (78)	60°C - 30	30	Leucocytozoon sp. leuB (KJ415277)
qLeuBR	AAATCCACCACAGACCCAA				
LankF	TGGATTTCTGCCGTGATCGT	18S rRNA (156)	60°C - 30	30	*Lankesterella valsainensis* (DQ390207)
LankR	ACAAGCCTGCTTGAAACACTCTATT				
TryF	ATGCACTAGGCACCGTCG	18S rRNA (121)	60°C - 30	30	*Trypanosoma avium* (KJ415280)
TryR	GGAGAGGGAGCCTGAGAAATA				

* Amplicon size in base pairs (bp); Cyt b = cytochrome B

† Temperature - time (seconds)

‡ Time (seconds); extension temperature was 60°C

§ GenBank accession numbers are indicated

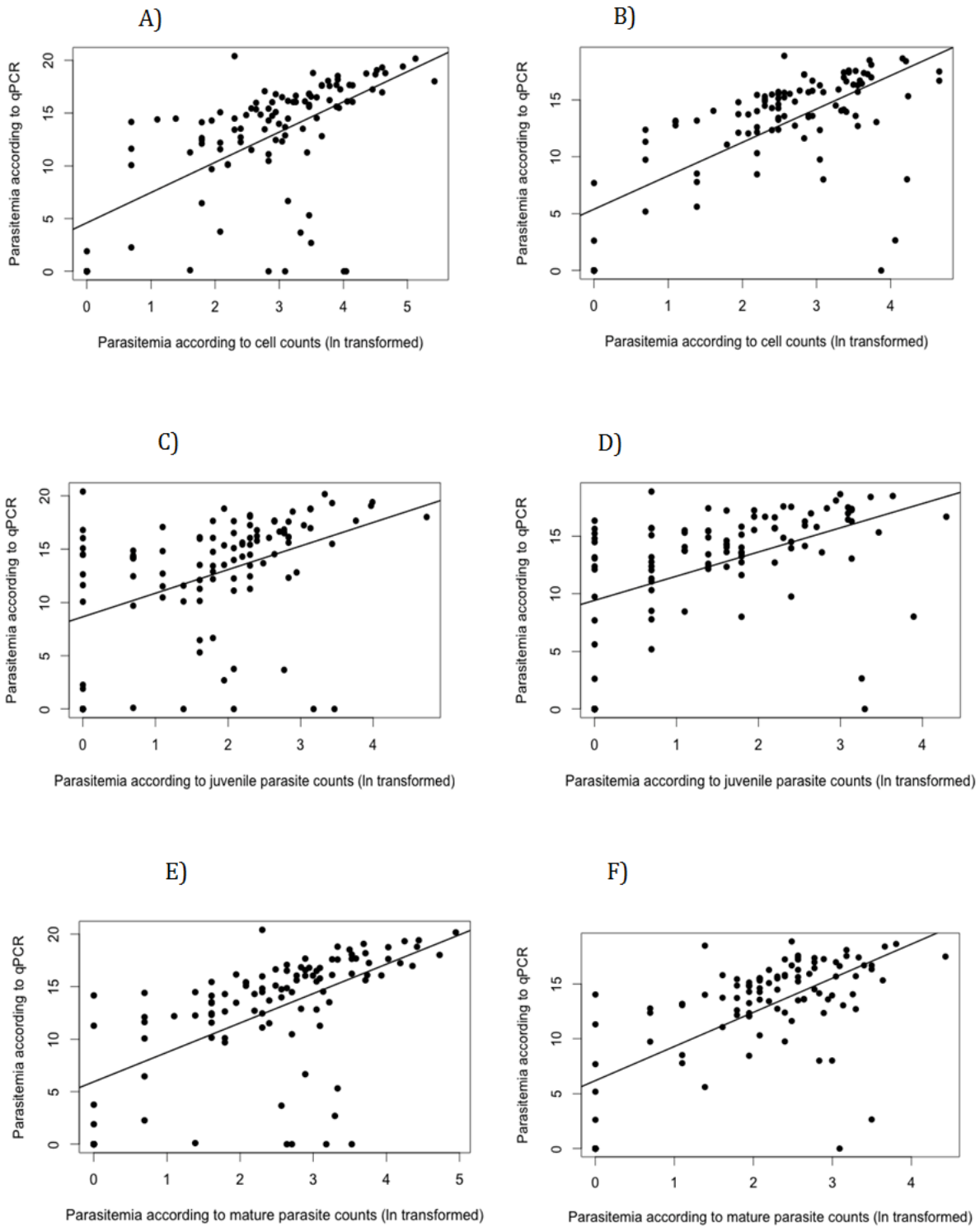

Figure 4. Correlation between *Haemoproteus* intensity of infection as calculated by the two methods: molecular vs. microscopic examination of blood smears. Initial samples (A,C,E) and final samples (B,D,F) are shown. A and B refer to total cell counts, C and D refer only to juvenile stages of the parasite, E and F refer only to mature stages of the parasite.

MODELS OF AVIAN VISION

'The slice of reality that we can see
is limited by our biology.'

—David Eagleman
'The brain: the story of you', 2015

Bird vision is very different from human vision; for instance, four single cone types are present in bird eyes whereas humans have only three (Cuthill 2006). But species vary in many other aspects in their visual system. The four single cone types for bird vision are categorized after the relative stimulation of wavelengths: ultra-short (UV), short (SW), medium (MW) or long (LW). The blue tit is a relatively well-studied bird in terms of its visual system, and data on the spectral sensitivity of each cone type is available in Hart et al. (2000). In fact, the blue tit is used as a model species for many other studies in bird coloration, as it seems broadly representative of many higher passerines with an ultraviolet shifted visual sensitivity for their UV cone type (Hart and Hunt 2007). The photon catch of an object is given by:

$$q_i = \int R_i(\lambda) S_i(\lambda) d(\lambda) ,$$

where q_i is the quantal catch of receptor type i, λ is the wavelength for the range over which the signal is measured (300 nm-700 nm for most birds), S_i is the spectral sensitivity of receptor i, $R(\lambda)$ is the spectrum of light entering the eye, and $d(\lambda)$ refers to the integration over the visible spectrum (following Endler and Mielke 2005; Stevens 2011 and references therein). In the blue tit, the percentages of double, LW, MW, SW and UV sensitive cones are 37, 20, 20, 15 and 8%, respectively (Hart et al. 2000). Oil droplets change the spectral sensitivity of cones (Cuthill 2006) and thus, values of spectral sensitivity are corrected using the method described in Hart et al. (2000).

Osorio et al. (1999) conducted behavioral experiments using domestic chicks (*Gallus gallus*) and found that all four single cone types were used in colour discrimination, and that their outputs seem to be connected in at least three opponent channels: 'UV versus SW', 'MW versus LW', and '(MW+LW) versus SW'. Thus, the calculations we used for hue were based on colour contrasts, in other words, the way that opponent colour channels broadly work. For the present blue tit population, hue was then calculated for the yellow breast feathers as the ratio of cone catch values for '(LW+MW+UV) versus SW'. Saturation is generally taken as the distance of an object from the achromatic center (Endler and Mielke 2005; Stevens 2011). To calculate saturation, we plotted the standardized single cone catch data for each individual (using relative cone catches to remove variations in absolute brightness) in avian tetrahedral colour space (Endler and Mielke 2005) and calculated the distance from the center of the colour space. Larger values indicate greater saturation.

PATERNITY ANALYSES

'In some species or populations,
extra-pair paternity is a phenomenon that cannot be ignored.'

—Emmi Schlicht and Bart Kempenaers
'From Genes to Animal Behaviour', 2011

Parents and nestlings were genotyped for eight microsatellite loci in order to explore paternity; information on microsatellites, primers and polymerase chain reaction (PCR) conditions are detailed in Table 2. PCRs were carried out in 10 µl volume using a QIAGEN Multiplex PCR Kit (Qiagen, Valencia, CA) and 20-50 ng of template DNA. Fluorescently labelled PCR products were separated on an AB3730 DNA analyser. Subsequently allele lengths were determined using Genemapper 4.0 software. For each

nestling the microsatellite genotypes were compared with its social father, and the offspring was assigned as extra-pair if there were at least two mismatches between the genotype of the social father and offspring. Extra-pair paternity (EPP) was assigned when one of the sampled males matched all of the offspring's paternal alleles. Paternity was assigned using Cervus 3.0 (Kalinowski et al. 2007). Maternity of the social female was confirmed by the microsatellite data for all nestlings. The mean exclusion probability of the eight markers was calculated for data on paternity from the 2012 and 2013 breeding seasons, both for the first (female) parent and for the second (male) parent (given the genotype of the first parent) (values are presented in **Chapters 3 and 4**).

Table 2. Microsatellite loci used in paternity analyses.

Locus	Fluorescent Label	PCR product length (bp)	Concentration (nM)*	Mix†	Reference
Pca8	Hex	250-450	50	1	Dawson et al. 2000
Pdo5	Fam	240-300	50	1	Griffith et al. 1999
PmaGAn2	Ned	190-260	20	1	Saladin et al. 2003)
7		170-240	20	1	Richardson et al.
Ase18	Hex				2000
Pca3	Hex	160-200	62	2	Dawson et al. 2000
Pca7	Fam	120-150	30	2	Dawson et al. 2000
Pocc6	Fam	220-260	62	2	Bensch et al. 1997
PmaTGAn45	Hex	300-390	18.5	2	Saladin et al. 2003

* Final concentrations for each forward and reverse primers

† Mix indicates which microsatellite loci were used in the same multiplex PCR reaction. The PCR settings were the same for both PCR mixes: initial denaturation at 95°C for 15 min; 35 cycles (denaturation at 94°C for 30 s, annealing at 55°C for 90 s, and extension at 72°C for 60 s); final extension at 60°C for 30 min.

TELOMERE SHORTENING ANALYSES

'Researchers eager to leap into telomere biology
should fully comprehend the potential methodological pitfalls of RTL.'

—S. Smith, C. Turbill and D.J. Penn
Heredity, 2011

For relative telomere length quantification measured by qPCR (RTL-PCR) a reference gene that is non-variable in copy number across the genome is needed (Smith et al. 2011). Primers used are shown in Table 3. Although some authors suggested that the zebra finches primers used in several telomere analyses were inappropriate for blue tit studies (Atema et al. 2013), this was not the case for our blue tit samples. The melting curves of the control gene cycles confirmed no primer-dimer non-specific amplification, and the efficiency was close to 2 for all PCR plates: E (plate GAPDH 1)=1.885, E (plate GAPDH 2)=1.945, E (plate GAPDH 3)=1.879; the slopes of the standard curves ranged from -3.649 to -3.460 with R^2 between 0.98 and 1.00. Similar results were obtained for the telomere analyses, with amplification efficiencies of E (plate1)=1.952, E (plate 2)=1.976, E (plate 3)=1.914; slopes for the standard curves ranging from -3.548 to -3.382, and R^2 between 0.98-1.00. The coefficients of variation for the GAPDH and telomere amplifications were less than 5% in all samples following Criscuolo et al. (2009). Sample level repeatability within and across plates was above 97.8% for GAPDH and telomere RTL-PCR.

Table 3. Primer sequences and PCR conditions for telomere shortening analyses.

Primers	Sequence 5'→ 3'	Gene (size)*	Annealing†	Extension‡	Species (specificity)§
GAPDHF	AACCAGCCAAGTACGATGACAT	12p chromosome (122)	56°C - 40	40	*Taeniopygia guttata* (AF255390)
GAPDHR	CCATCAGCAGCAGCCTTCA				
Tel1b	CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGG		56°C - 30	40	-
Tel2b	TTTGGGTT				
	GGCTTGCCTTACCCTTACCCTTACCCTTACCC				
	TTACCCT				

* Amplicon size in base pairs (bp)

† Temperature - time (seconds)

‡ Time (seconds); extension temperature was 60°C

§ GenBank accession numbers are indicated

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PART I

PARASITES AND COLOUR

Chapter 1

'The fishermen know that the sea is dangerous and the storm terrible, but they have never found these dangers sufficient reason for remaining ashore.'

—Vincent van Gogh

This chapter reproduces entirely the manuscript:

E.P. Badás, J. Martínez , J. Rivero-de Aguilar , Carlos Ponce , M. Stevens and S. Merino. Colour change between seasons in a structural ornament is related to individual quality and mating patterns in the blue tit.

Colour change between seasons in a structural ornament is related to individual quality and mating patterns in the blue tit

E.P. BADÁS, J. MARTÍNEZ, J. RIVERO-DE AGUILAR, CARLOS PONCE, M. STEVENS, AND S. MERINO

Abstract Carry-over effects refer to processes that occur in one season and influence fitness in the following season. In birds, two costly activities, namely reproduction and moult, are restricted to a small time window, and sometimes overlap. Thus, colour deposition in the newly moulted feathers is likely to be affected by the costs of reproduction. Using models of bird vision and recaptured individuals from two consecutive seasons, we investigated male colour change in a free-living population of blue tits (*Cyanistes caeruleus*). We related feather colouration after the moult to the intensity of blood parasite infections experienced during reproduction. In the following spring, we explored mating patterns with respect to previous changes in feather colour. During reproduction, males that were less intensely infected by the malaria parasite *Plasmodium* showed a more pronounced decrease in white cheek saturation in winter, which may indicate higher feather quality. Additionally, increased brightness in the white cheek was associated to better body condition while breeding. In the following season, males with brighter cheeks paired with brighter females. The male's partners from both breeding attempts were found to be visually discriminable in their white cheek brightness. These results suggest that the conditions experienced during reproduction are likely to affect feather colouration obtained during moult, at least in the white patch. High quality individuals that allocate resources efficiently during reproduction could increase future reproductive success through variation in mating patterns. Carry-over effects from reproduction might extend not only to the non-breeding phase, but also to the following breeding season.

Keywords Achromatic colouration, body mass, life-history theory, sexual selection, signalling, structural colouration

INTRODUCTION

A central tenet of life-history theory is that resources allocated to current reproduction are traded-off against self-maintenance and future reproductive output (Stearns 1992; Metcalfe and Monaghan 2001). In birds, a growing body of research has investigated the mechanisms behind the costs of reproduction (reviewed in Harshman and Zera 2007; Blount et al. 2016), and the costs *per se*, which can come as a reduction in survival (Santos and Nakagawa 2012), for example, through accelerated ageing (Bize et al. 2009; Badás et al. 2015). Others have focused on the downstream effects of non-breeding season processes on reproduction, commonly known as 'carry-over effects' (Gunnarsson et al. 2006; Robb et al. 2008; Sorensen et al. 2009). Fewer studies have explored the effects of processes that occur during the breeding season on the non-reproductive season, when these, in fact, could influence the outcome during the following breeding event (Harrison et al. 2011).

Reproduction can exert changes in individual's post-breeding activities such as the moult. Moulting is an energetically demanding process (Griggio et al. 2009), because it encompasses physiological (i.e. altering multiple stress response pathways, Merino and Barbosa 1997) and metabolic costs (an increase in 30% of metabolic rate) (Cyr et al. 2008). Many birds initiate the post-nuptial moult while still raising young (Jenni and Winkler 1994), but because these activities are highly demanding, they should be separate in time. Indeed, passerines that were already moulting while breeding had reduced fledgling success (Sanz 1999; Hemborg et al. 2001; Morales et al. 2007). Delayed reproduction can compromise the time allocated for moulting, thus reducing feather quality, as has been reported in starlings (*Sturnus vulgaris*), (Dawson et al. 2000). Furthermore, Nilsson and Svensson (1996) showed that blue tits (*Cyanistes caeruleus*) that delayed moult had higher thermoregulatory costs in the following winter, and this resulted in reduced over-winter survival and breeding success the following year. Thus,

the effects in feather synthesis become evident when reproductive effort exceeds what individuals were prepared to sustain. Additional information is needed on whether the individuals' status during reproduction has an important bearing on feather quality. Yet, data on recently moulted birds in free-living populations are scarce because re-trapping the same individuals repeatedly is difficult (Dawson et al. 2000).

Reproduction can also affect immunocompetence (Hanssen et al. 2003). In temperate regions, bird populations usually suffer from chronic blood parasite infections with relapses during the breeding season (Valkiūnas 2005), and the negative effects that these parasites exert on the host are well studied (Merino et al. 2000; Martínez-de la Puente et al. 2010; Asghar et al. 2015). Strong immune responses may have negative effects on the moult, decreasing the amount of resources available and resulting in a delayed onset of post-nuptial moult (Sanz et al. 2004, but see Moreno et al. 2001). In addition to this, experimental studies have shown that certain aspects of structural colouration can signal food stress (Siefferman and Hill 2005) or acute parasite infection during the moult (Doucet and Montgomerie 2003).

The costs of reproduction on feather quality could be assessed through colouration, because colours are incorporated to new feathers during moult (Hill and McGraw 2006). For example, in eiders, it has been suggested that reproductive females with reduced lymphocyte levels may suffer from infections in their following moult, which could reduce the reflectance of the white plumage bands (Hanssen et al. 2006). In blue tits, experimentally increasing the cost of reproduction produced changes in feather colouration in two ornaments in the year following manipulation (Doutrelant et al. 2012). In fact, because plumage colours are produced through different metabolic pathways depending on its nature (structural or pigmentary), they are subject to different constraints that may convey information to prospecting mates (Hill 2006). Other mechanisms, such as soiling (Fitzpatrick 1998) or feather degrading bacteria (Delhey et al.

2006) can also explain changes in colour during the season, but to date, no study has evaluated these changes with respect to parasitic infections during reproduction.

In this study, we investigated colour change in plumage patches that differ in their main mechanism of colour production (structural, pigmentary, or both), and related theses changes to parasitic infections during reproduction and to other breeding parameters. Structural colouration results from the particular feather microstructure that scatters light in coherent on incoherent levels (Prum 2006), and in the blue tit, it is seen in plumage patches like the blue crown and the white cheek. Carotenoid pigments are obtained from food and deposited in feathers producing yellow colours (McGraw et al. 2002), as seen in the blue tit's breast. We also measured patches for which less information is available in the literature, such as the olive-green base of the tail. This plumage patch has been described as sexually dichromatic in nestlings (Johnsen et al. 2003), and it is likely to be relevant for sexual selection in the blue tit. Besides, green colour is generated by the combination of a structural blue component and yellow pigments (Prum 2006). Studies investigating several ornaments simultaneously are increasing (Doucet and Montgomerie 2003; Hegyi et al. 2007; Galván 2010), but changes in feather colour in multiple ornaments are understudied. Furthermore, adult blue tits undergo a complete moult once a year, hence the plumage achieved during moult will be carried until the end of the next breeding season (Nilsson and Svensson 1996). For this reason, the carry-over effects of reproduction on moult and feather colouration may have an effect on mating patterns in the following reproductive event.

First, we aimed at relating individual status during reproduction to colour change. Individual quality during the breeding season was evaluated by measuring body condition during the highly demanding nestling provisioning phase and the intensity of infections by several blood parasites (avian malaria and malaria-like parasites). We expect reproductive costs to negatively affect colour change in poor quality individuals. Although unexplored

so far, an individual may gain higher quality partners in the following season if performance in the previous reproductive event was high (see Griggio et al. 2009). On this basis, the second aim of this study was to investigate the effects of colour change and breeding parameters on matting patterns in the consecutive breeding season. Changes in the mate's feather colouration from one season and the following were confirmed by discrimination models that are sensitive to avian vision (Endler and Mielke 2005; Stevens 2011).

METHODS

Study site and sampling

Data were collected during the spring and winter of 2013 (hereafter season 1), and the spring of 2014 (hereafter season 2) on a free-ranging population of blue tits breeding in a deciduous forest of Pyrenean oak (*Quercus pyrenaica*), in the vicinity of Valsaín (Segovia), central Spain (40°53'N, 4°01'W, 1200 m.a.s.l.), where 300 wooden nestboxes have been in place since 1994 (Fargallo & Merino, 1999). Breeding birds in seasons 1 and 2 were caught at the nestbox during chick provisioning (when nestlings were three days old, hatching date = day 0), whilst birds caught in the winter of season 1 were trapped using song-baited mist nets. At every sampling occasion 2-3 nets (24-36 m each) were set up for 1-2 hours and then they were moved to a different location within the vicinity of the deciduous forest. Bird captures took place in 6 days (dates: 1, 2, 3, 9, 10 and 24 of November 2013) during 4-5 hours each day depending on climatic conditions. Unringed birds were individually marked with a numbered aluminium leg-ring. First-years were identified (if age not known from ringing records) by possession of distinctive, non-adult greater wing coverts (Svensson 1992). We also recorded tarsus length to the nearest 0.01 mm using callipers and weight to the nearest 0.1 g using an electronic balance. These measurements were used to calculate individual body mass, corrected by regression for

body size (tarsus length) and time of day by using the equation from Senar (2002). We also measured feather colour reflectance on four different patches in males and females: breast, cheek, crown and base of the tail. Colour spectra were collected both in spring and winter using a spectrophotometer (Ocean Optics Inc., Dunedin, FL, USA) connected to an Ocean Optics fibre-optic reflection probe. The probe was made up of seven optical fibres that were illuminated by a Pulsed Xenon Light Source (Jaz-PX lamp) and it was inserted in a miniature black chamber that acted as holder and excluded ambient light. The equipment was calibrated with a flat white standard (Ocean Optics) prior to each patch measured. The probe was lifted between repeated measurements within a body region. Reflectance data from 300 to 700 nm were undertaken at 90º incidence and 3 mm from the feather surface over an illuminated circular area approximately 1 mm in diameter. Each spectrum was an average of three scans and was calculated relative to the reflectance produced by the white standard and a dark current.

At the breeding season 1 we took a blood sample via the brachial vein. One drop of blood was stored on an FTA card (Whatman, UK) for molecular analyses (parasitological analyses, see below).

Moult stage was recorded both at the breeding season and winter captures. One individual had already started moult when captured during breeding season 1 (as of 28th of June). However, this male was not recaptured in winter (season 1). By the time they were recaptured in November of season 1, all individuals had already finished moulting. None of the birds used in this study had started moulting when captured at nestling age three in breeding seasons 1 or 2.

Parasite quantification (spring, season 1)

For all samples, DNA was extracted from blood using a standard ammonium-acetate protocol and stored at -20ºC. This DNA solution was then purified using silica

filters to obtain a higher quality DNA (NZYGel pure, NZYtech, Lda. -Genes and Enzymes). DNA samples were quantified by spectrophotometry and adjusted to the same concentration (10ng/uL). We detected and quantified the following parasites using quantitative PCR (qPCR) with SYBR green (SYBR Selected Master Mix, Applied Biosystems) to amplify a fragment of the cytochrome B or 18S rRNA genes using a pair of species-specific primers for each parasite: *Haemoproteus majoris* haplotype cyan2, *Plasmodium* spp. haplotype cyan1, *Lankesterella valsaininesis*, and *Leucocytozoon* spp. haplotypes leuA, leuA1 and leuB. The variable *Leucocytozoon* A includes haplotypes A and A1 (see Badás *et al.*, 2015 for more information on the primers used).

Models of bird vision (seasons 1 and 2)

To model the UV-sensitive (UVS) blue tit visual system, we used their known photoreceptor spectral sensitivities (Hart et al. 2000) and calculated the relative quantum (photon) catch values for the four single cones, used in colour vision, and the double cones, used in luminance vision (Endler and Mielke 2005; Stevens et al. 2009). From this, we extracted hue, saturation, and luminance variables for each colour patch (Endler and Mielke 2005; Stevens et al. 2009). Although hue and saturation colour variables may not necessarily relate to colour perception in birds, avian visual models that incorporate cone sensitivities of the bird's retina and light conditions, have proved to be the most widely approach used to model avian colour vision and colouration (Stoddard and Prum 2008; Kemp et al. 2015). Luminance refers to the perceived lightness of a patch (brightness); so we simply used the double cone photon catch values. Saturation refers to the amount of colour compared with white light, and it was obtained by plotting the standardized single cone catch data for each individual in avian tetrahedral colour space (Stevens et al. 2009) and calculating the distance from the centre of the colour space (following Endler and Mielke 2005). To calculate hue or colour type, we derived colour channels based on using ratios from the photon catch outputs for each patch (see **General Methods Chapter**). This

approach is broadly inspired by the way that opponent colour channels work in vision in encoding antagonistic colour types (Osorio et al. 1999) and is based on recent work following the same methods (Komdeur et al. 2005; Spottiswoode and Stevens 2011; Stevens et al. 2014). Hue was not calculated for the white cheek because this is an achromatic ornament.

Following calculation of photon catches we determined colour contrasts using a model of visual discrimination that accurately predicts discrimination behaviour in observers (Vorobyev et al. 1998). By using the single cones we extracted colour differences (Vorobyev et al. 1998), and using the double cones we obtained luminance (achromatic) differences (Siddiqi et al. 2004). When modelling, we used the retinal single cone proportions of the blue tit available in the literature (long wave = 1.00, medium wave = 0.99, short wave = 0.71, and UVS = 0.37; Hart et al. 2000). The results of these models are expressed in 'just noticeable differences' (JND), where generally a JND of less than 1.00 indicates that two stimuli are indistinguishable; values between 1.00 and 3.00 should be difficult to discriminate except under optimal viewing conditions; and larger values allow increasingly easy discrimination (Siddiqi et al. 2004).

Then, for each individual and colour patch, we evaluated the change of colour between winter (season 1) and the following season (season 2) by calculating chromatic and achromatic colour contrasts and reporting JND scores. Finally, for a subsample of males (N=13, see below), we obtained JND scores describing the differences between their female partners from breeding seasons 1 and 2 and used these values in subsequent analyses.

Statistical analyses

All analyses were performed in R v.3.1.3 (R Foundation for Statistical Computing, Vienna). We used saturation and luminance (not hue) for each colour patch analyses.

Yellow hue and saturation were highly correlated (Spring: r = 0.87, p<0.001; Winter: r = 0.81, p<0.001), and we chose to use saturation rather than hue as it most consistently reflects feather carotenoid content across species (Saks et al. 2003; McGraw and Gregory 2004). Hue and saturation were also correlated in the blue crown (Spring: r = 0.99, p<0.001; Winter: r = 0.98, p<0.001) and blue-green tail (Spring: r = 0.91, p<0.001; Winter: r = 0.90, p<0.001) plumage, and again, we used saturation rather than hue. In winter, females had lower recapture probability than males (Chi-sq=12.63, df=1, p<0.001, N=40), probably because the method of capture using song baited mist nest might attract males preferentially. Thus, females were not included in the analyses (data on breeding parameters and parasite infection was available only for one female). From 78 breeding pairs in the breeding season 1, we were able to recapture 21 males in winter (annual survival rates of adult blue tits are similar in other European populations, see Dhondt et al. 1998). Due to limiting blood volumes for molecular analyses on 4 individuals and 2 individuals for which colour data could not be obtained because of measurement error, data on breeding parameters and parasitic infections during reproduction was available for 15 individuals that were included in the analyses.

To explore the differences in colour between spring and winter (season 1), we fitted a linear mixed model for each feather patch with one of the colour variables as the response variable and sampling occasion as a fixed factor; individual identity was used as a random factor in order to control for repeated measures on the same individual. Models also included age, date of winter sampling, and several parameters from the breeding season: hatching date, body mass, and parasite infection intensity by four blood parasite species (see parasitological analyses above). Parasite intensity variables were cubic root transformed and classified by quantiles in order to categorise data in two meaningful groups: low and high intensity of parasitic infections. Because we were interested in the change in feather colour with respect to individual status at the breeding season, winter body mass was not included in the analyses. Moreover, spring body mass was correlated

to winter body mass (t=2.84, df=17, correlation coefficient: r = 0.57, p=0.01). Date of sampling was incorporated in the models to account for its effect on feather colouration and infection probability. Age was obtained from previous ringing records and codified into a 3 level score as follows due to reduced sample size of older individuals: 1=first-years (N=9), 2=second-years (N=6), 3=three-years and older (N=6). Each model included the interaction between sample (spring or winter season 1) and one parasite species at a time due to limited sample size (we specifically tested for the interaction, as seen in Knowles et al. 2010). The final model was selected based on AIC (Akaike Information Criterion) via its corrected version for small sample sizes (AICc, Sugiura 1978). When the difference in AIC between two or more models was less than 10 AIC units (**Δ**AIC<10), all models were considered because these are thought to be reasonably well-fitted models (Bolker et al. 2009). In order to quantify the relative importance of individual variables we calculated model weights (Johnson and Omland 2004) from all models tested in the analyses (including those with a difference in AICc higher than ten units) (see Table A1 in the Appendix for details on the selected models). This was further confirmed by estimates of significance for full vs. null models that were obtained by parametric bootstrap procedures due to reduced sample size ('PBmodcomp' command from the R package pbkrtest following Halekoh and Højsgaard 2014). Model parameters and 95% confidence intervals for the main effects were calculated from 1,000 bootstrapped iterations derived with 'bootMer' (from the R package lme4, Bates et al. 2014) for all significant models.

Finally, we explored whether changes in male colour variables between seasons 1 and 2 explained better performance in season 2. Out of the 21 individuals captured between spring and winter of season 1, 13 males were recaptured again in season 2. We calculated the rate of colour change between breeding seasons for patches where we had previously found significant colour change: $(C_2-C_1)/C_1$, where C_1 refers to colour in season 1 and C_2 to colour in season 2. Then, colour change was related to: (i) clutch size change between seasons 1-2, (ii) hatching date change between seasons 1-2, and (iii) the JND

scores describing perceptible differences in colour between female partners in seasons 1-2. The relationship between male colour change and other breeding parameters (i.e. number of fledglings) was not be checked because a post-hatching experiment conducted in season 2 could potentially affect these parameters. We aimed to look at trends shared between colour variables by using Pearson correlations, instead of using more complex modelling with such reduced sample size. In relatively small samples like the present one, general assumptions about the structure of the population (i.e. normality) can be violated, whereas non-parametric bootstrapping allows us to compute new parameter estimates without making assumptions on the form of the population (Freedman 1981). Thus, we further complemented the significant results obtained by Pearson correlations through robust regression models or with those calculated from 1,000 bootstrapped iterations derived from resampling with the function 'bootCase' (from the R package car, Fox and Weisberg 2011).

RESULTS

Colour change from spring to winter in season 1

Plumage colouration in the white cheek changed significantly before and after the moult (Table 1). The overall trend was that individuals decreased saturation in their white cheek feathers after the moult, but males that were more intensely parasitized by *Plasmodium* during the breeding season decreased saturation significantly less (Fig.1, for graphical purposes we represent mean changes between sampling occasions). On the contrary, saturation in their yellow breast feathers increased after the moult, although this increase was marginally non-significant (Table 1, p=0.08). Such marginal increase was unaffected by parasite loads or other parameters during the breeding season. However, irrespective of sampling occasion, males with more saturated yellow breasts tended to harbour marginally more *Leucocytozoon* A parasites in spring (Table 1, p=0.09). No significant change was detected for the blue crown and the blue-green tail (Table 1).

However, there was a trend that males that were more parasitized by *Leucocytozoon* A developed more saturated blue feathers after moulting (Table 1, p=0.08). Finally, males with more saturated blue crowns tended to have marginally higher body mass, irrespective of sampling occasion (Table 1, p=0.09).

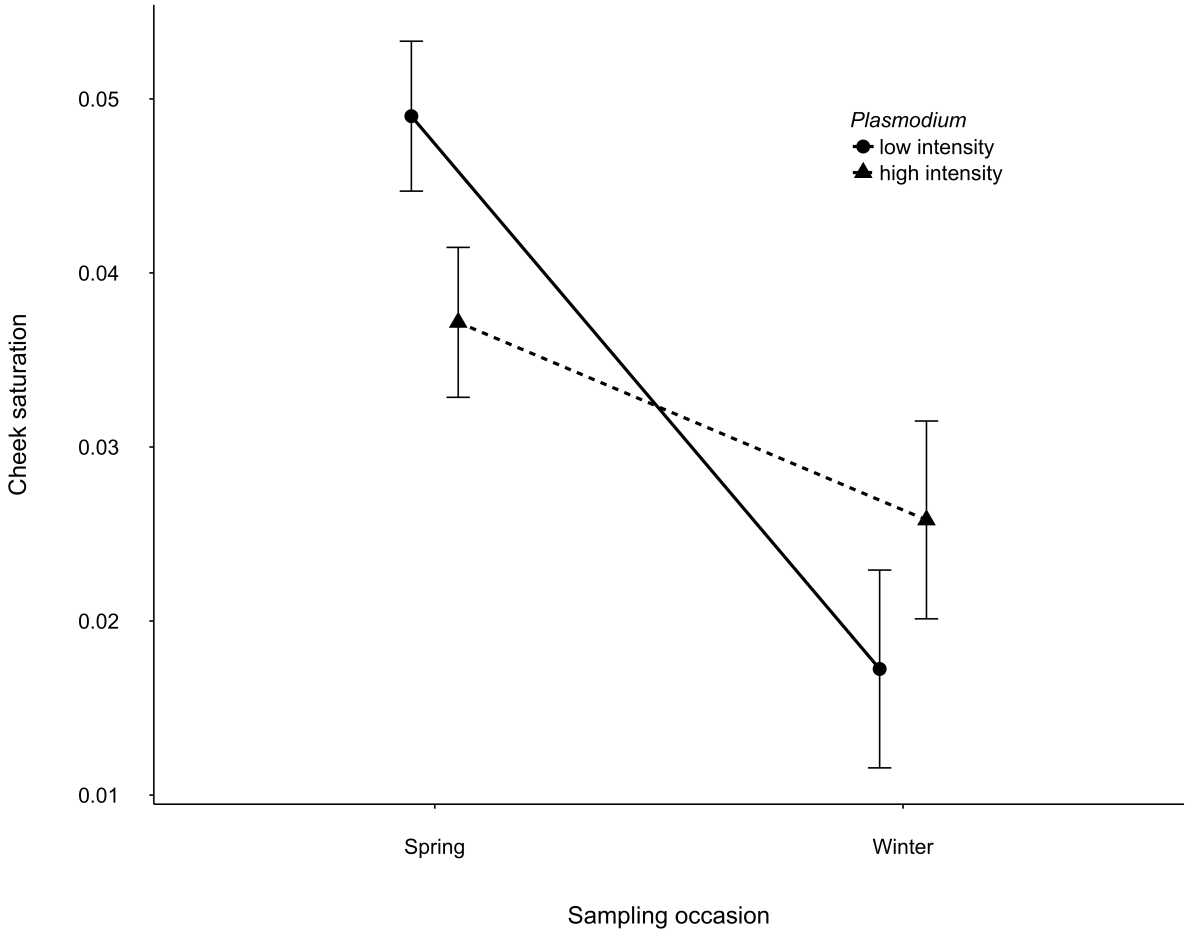

Figure 1. Chromatic change between spring and winter in the white cheek in relation to *Plasmodium* infection during spring. Bars indicate standard errors.

Table 1. Results for the best-fit mixed effects models describing chromatic change from spring to winter (season 1). Sample sizes for each set of models are provided. Significant parameter estimates are indicated, i.e. variables for which their unconditional 95% confidence interval (CI) after bootstrapping did not cross zero. Codes: (S)=body mass in the Spring sample, SE=standard error, ES=effect size expressed as Cohen's D, N=sample size.

Model	Predictors	Estimated parameters ± SE	Student's T	P-value	ES	N
Cheek						
1	**Sample**	**-0.41±0.07**	**-5.54**	**<0.0001**	**1.42**	30
	Plasmodium	0.024±0.12	0.20	0.84	0.05	
	Sample x *Plasmodium*	**-0.005±0.002**	**-2.34**	**0.036**	**0.57**	
2	Body mass (S)	0.004±0.007	0.64	0.53	0.17	
	Sample x body mass (S)	-0.002±0.005	-0.33	0.75	0.1	
3	*Leucocytozoon* B	0.00005±0.003	0.01	0.99	0.008	
	Sample x *Leucocytozoon* B	0.003±0.002	-1.33	0.2	0.36	
4	*Leucocytozoon* A	-0.001±0.004	-0.27	0.79	0.07	
	Sample x *Leucocytozoon* A	-0.003±0.003	-1.06	0.31	0.3	
5	*Haemoproteus*	0.002±0.003	0.54	0.6	0.15	
	Sample x *Haemoproteus*	-0.001±0.003	-0.46	0.65	0.13	
1	Sample	0.11±0.06	-1.87	**0.08**	0.48	30
	Body mass (S)	-0.004±0.008	-0.52	0.61	0.14	
	Sample x body mass (S)	-0.009±0.005	-1.64	0.12	0.42	
2	*Leucocytozoon* A	-0.007±0.004	-1.82	**0.09**	0.49	
	Sample x *Leucocytozoon* A	0.004±0.003	1.5	0.16	0.4	
3	*Haemoproteus*	0.005±0.004	1.19	0.26	0.32	
	Sample x *Haemoproteus*	-0.004±0.003	-1.31	0.21	0.35	
4	*Leucocytozoon* B	-0.006±0.004	-1.5	0.16	0.41	
	Sample x *Leucocytozoon* B	-0.003±0.003	0.88	0.39	0.23	
5	*Plasmodium*	-0.005±0.004	-1.43	0.18	0.39	
	Sample x *Plasmodium*	0.002±0.003	0.77	0.45	0.2	
Tail						
1	Sample	-2.25±1.41	-1.59	0.14	0.52	28
	Body mass (S)	0.29±0.18	1.65	0.13	0.54	
	Sample x body mass (S)	0.22±0.13	1.63	0.13	0.54	
Crown						
1	Sample	-0.04±0.11	-0.42	0.68	0.12	30
	Body mass (S)	0.02±0.01	-1.78	0.1	0.58	
	Sample x body mass (S)	0.005±0.01	0.52	0.61	0.14	
2	*Leucocytozoon* A	-0.03±0.008	-0.35	0.73	0.09	
	Sample x *Leucocytozoon* A	-0.009±0.005	-1.9	**0.08**	0.56	

Patterns of achromatic change from spring to winter in season 1 were also affected by individual quality during the spring. Brightness in the white cheek increased after the moult, and this increase was positively affected by higher body mass during the breeding season (Table 2, Fig.2). We also detected a marginally non-significant increase in yellow

breast and green tail brightness after the moult (Table 2). Males that were more parasitized by *Haemoproteus* tended to grow marginally brighter yellow breasts (Table 2, p=0.08) and marginally brighter green tails (Table 2, p=0.08). Intense infections by the parasite *Leucocytozoon* B during the breeding season had a marginally non-significant negative effect in brightness in the green tail in winter (Table 2, p=0.07). Finally, no significant change was detected for the blue crown brightness (Table 2).

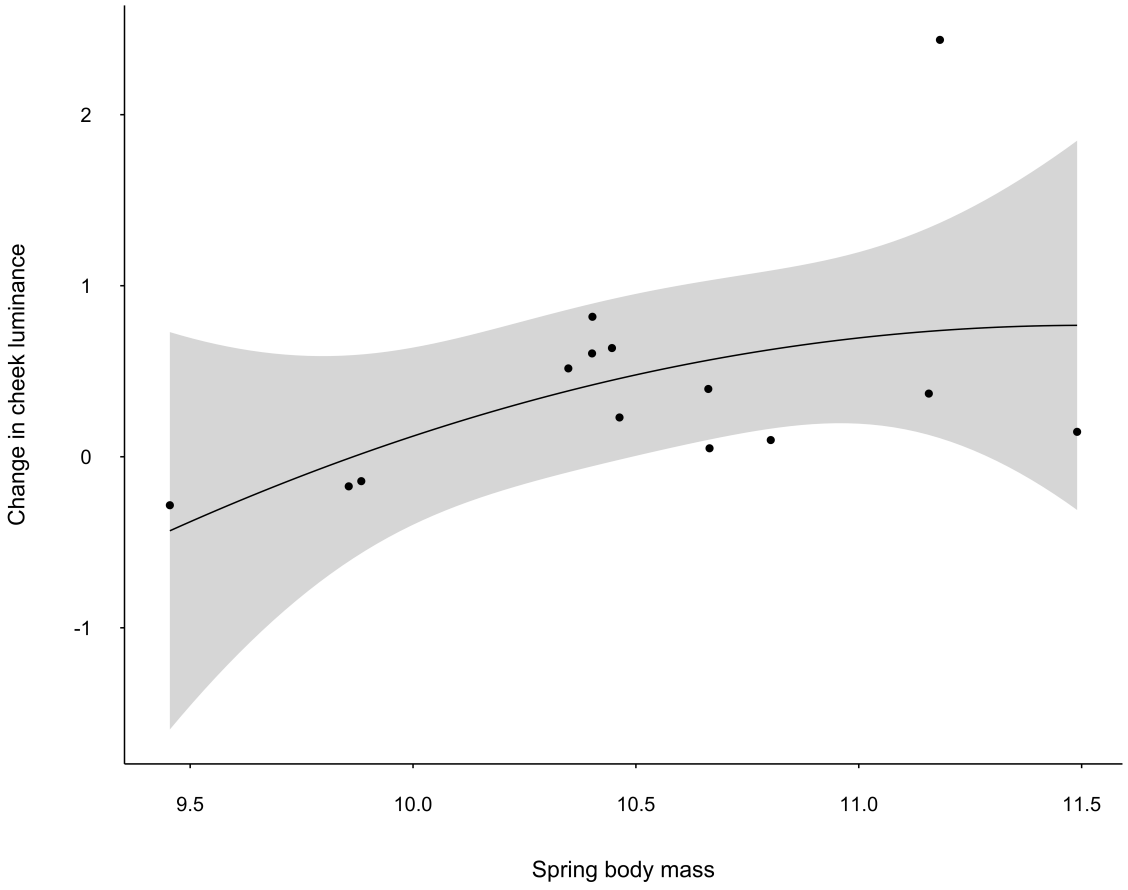

Figure 2. Achromatic change in the white cheek in relation to spring body mass ($F_{2,10}$=4.67, p-value=0.0382, R^2=38.54%, N=14). Body mass (g) is expressed as the corrected body mass following Senar (2002). Regression line and ±95% confidence intervals (shaded area) are shown.

Table 2. Results for the best-fit mixed effects models describing achromatic change from spring to winter (season 1). Sample sizes for each set of models are provided. Significant parameter estimates are indicated, i.e. variables for which their unconditional 95% confidence interval (CI) after bootstrapping did not cross zero. Codes: (S)=body mass in the Spring sample, SE=standard error, ES=effect size expressed as Cohen's D, N=sample size

Model	Predictors	Estimated parameters ± SE	Student's T	P-value	ES	N
Cheek						
1	**Sample**	**-0.57±0.22**	**-2.57**	**0.023**	**0.67**	30
	Body mass (S)	0.017±0.03	0.49	0.63	0.12	
	Sample x body mass (S)	**-0.058±0.02**	**2.74**	**0.017**	**0.72**	
2	*Leucocytozoon* A	-0.012±0.02	-0.68	0.51	0.2	
	Sample x *Leucocytozoon* A	-0.002±0.005	-0.33	0.1	0.54	
3	*Leucocytozoon* B	0.0012±0.02	0.07	0.95	0.02	
	Sample x *Leucocytozoon* B	0.02±0.01	1.76	0.1	0.51	
1	Sample	0.013±0.006	2.18	**0.05**	0.76	28
	Haemoproteus	0.006±0.007	0.99	0.34	0.33	
	Sample x *Haemoproteus*	-0.011±0.006	-1.95	**0.08**	0.67	
Tail						
1	Sample	-0.03±0.002	-1.84	**0.09**	0.64	28
	Leucocytozoon B	0.002±0.002	0.72	0.49	0.24	
	Sample x *Leucocytozoon* B	-0.004±0.002	-1.96	**0.07**	0.71	
2	*Haemoproteus*	0.002±0.002	0.72	0.48	0.02	
	Sample x *Haemoproteus*	0.004±0.002	1.89	**0.08**	0.7	
Crown						
1	Sample	-0.13±0.11	-1.2	0.25	0.35	30
	Body mass (S)	-0.005±0.02	-0.32	0.76	0.11	
	Sample x body mass (S)	0.01±0.01	1.19	0.26	0.35	

Male colour, female partner and breeding parameters in season 2

Because we found significant differences in cheek colour between samples before and after moult (spring vs. winter season 1, see Tables 1 and 2), we used these variables in subsequent analyses for season 2. There were no significant associations between: (i) change in cheek saturation between seasons and breeding parameters in season 2 (all p-values>0.5), (ii) change in cheek saturation between seasons and JND scores for female differences between seasons (all p-values>0.5), or (iii) change in cheek brightness

between seasons and breeding parameters in season 2 (only hatching date and clutch size, see Methods section, all p-values>0.5). However, we found that males that were already brighter in their white cheek feathers in season 1 changed less between seasons (t=-5.55, df=19, correlation coefficient: r = -0.79, p-value<0.0001).

Additionally, when the increase in cheek brightness after the moult was more pronounced, those males paired with females that differed significantly in the luminance JND scores when compared to the female pair from season 1 (bootstrapped estimate=0.089, sup. 95% CI=0.18, inf. 95% CI=0.003, R^2= 0.425, $F_{1,9}$=8.39, p-value=0.018, N=13, effect size ES=1.93, Fig. 3). In other words, when males increased cheek brightness after the moult, their female pair was significantly more ornamented than the female pair they had in the previous season, according to an avian vision model based on blue tit perception. Indeed, the male's partner in season 2 had significantly brighter cheeks than the same male's partner in season 1 (robust regression model R^2=0.39, Chi-sq=6.17, df=1, p-value=0.013, N=13, effect size ES=1.9).

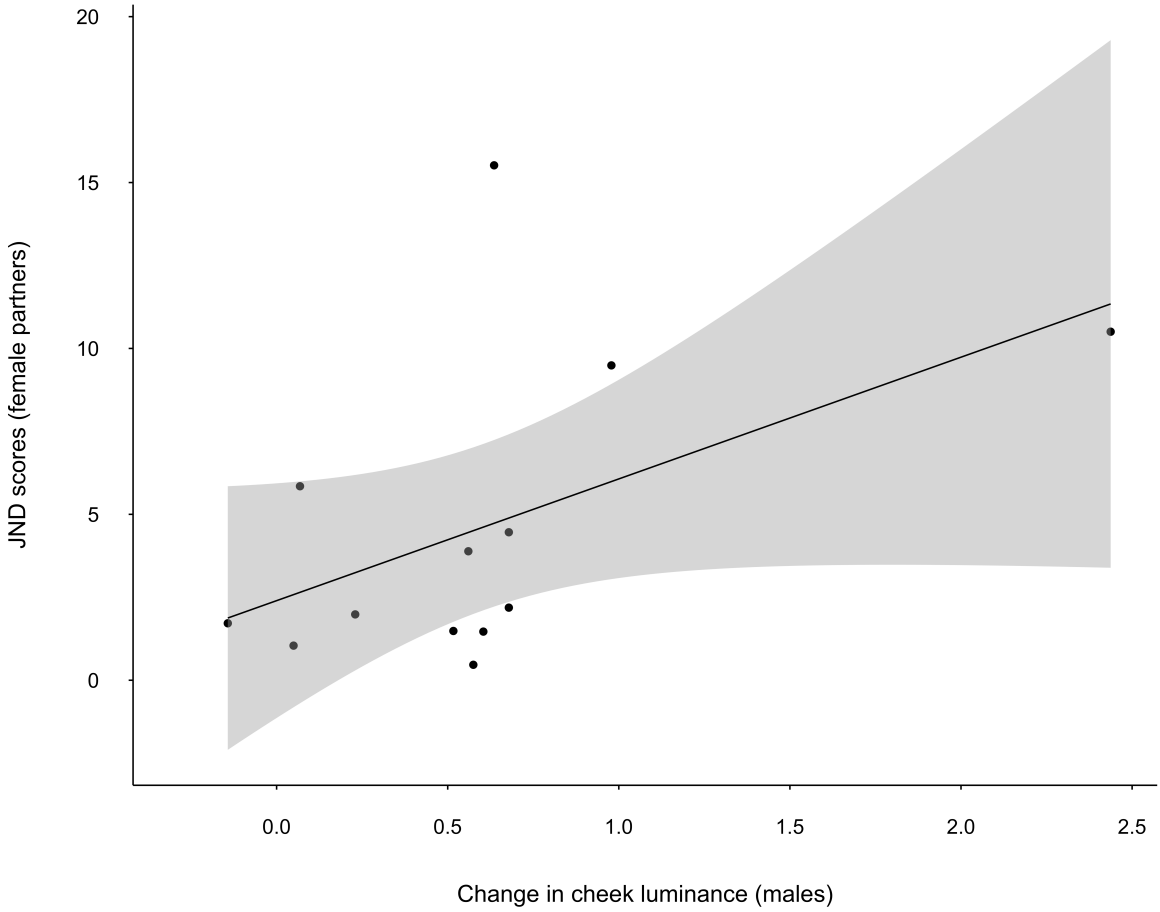

Figure 3. Change in male white cheek luminance between seasons in relation to female partners JND scores ($F_{1,9}$=8.39, p-value=0.018, R^2= 42.5%, N=13). Female JND scores were obtained as achromatic colour differences between the male partner in season 1 vs. season 2. Regression line and ±95% confidence intervals (shaded area) are shown.

DISCUSSION

This study investigated feather colour change in relation to breeding characteristics from the previous reproductive season. We offered correlational evidence that, in blue tits, colour change in the white cheek was related to body mass and the intensity of *Plasmodium* infections while breeding. Additionally, males with a more pronounced increase in white cheek brightness paired with brighter females in season 2 when compared to the females they paired with at the previous reproductive event. The

change in structural white colouration in the blue tit was, thus, related to pair formation in the consecutive breeding season.

Brightness in unpigmented feathers depends on feather structure (Prum 2006). For example, a detailed analysis of white feathers of the rock ptarmigan (*Lagopus mutus*), demonstrated that the brightness was produced by large, randomly organized air vacuoles in the barbules; and that these vacuoles were absent in species with less bright white feathers (Dyck 1976). Structural organization in feathers (i.e. the regularity of nanostructures of keratin and air vacuoles) may be costly to produce (Prum 2006). In the blue tit, the particular arrangement of barbules in the white cheek could be related to individual status during reproduction, because feathers are moulted immediately after the breeding season (Svensson and Nilsson 1995). In this study population, male blue tits that were heavier during the breeding season (season 1) might have started the moult with more resources to allocate into more complex feather structures. In fact, it has been suggested that parents that are in better physical condition have higher survival rates (Perrins and McCleery 2001) and are probably more efficient in food provisioning during reproduction (Lambrechts et al. 2004).

Other authors have reported no relationship between body condition and white structural colour in blue tits. However, in that case, plumage reflectance and body mass were measured in yearlings (raised in aviaries) after they underwent their first moult into adult plumage (Peters et al. 2011), while in the present study change in feather colour was related to body condition in the previous season. This could explain the different results obtained in both studies. Another study in males of the closely related great tit (*Parus major*) found a positive relationship between reflectance in the white cheek and winter body condition (Galván, 2010). In this study, spring and winter body mass were correlated, so male blue tits that increased cheek brightness were also in better body condition during winter. Passerines might signal body condition during winter because

this may have great relevance for the subsequent breeding season, as explained above. Indeed, in other passerines, there is correlational and experimental evidence that the information content of the white cheek is important for winter social interactions and signalling dominance rank (Mennill et al. 2003; Ferns and Hinsley 2004), and that it predicts reproductive success in the following breeding season (Doucet et al. 2005). In blue tits, dominance in winter has been related to lower winter mortality, better access to food resources and higher probability of establishing a territory in spring (Smith and Nilsson 1987); but the relationship between dominance rank and cheek brightness remains to be confirmed in our population.

Previous studies have found associations between condition and colour in the structural blue and green in other species (Siefferman et al. 2007; Budden and Dickinson 2009), but none analysed colour data with the use of avian visual models. Peters et al. (2011) used units that are relevant to the visual perception of birds, and reported no relationship between body mass and crown colouration in blue tits, which is in agreement with our findings. Patches like the blue crown, the blue-green tail, or the yellow breast, did not appear to be condition-dependent in males in this study. However, in another breeding season we found that heavier blue tit females were less saturated in their yellow breast feathers (Badás et al 2017). Other studies have suggested that different ornaments can provide different pieces of information in males (McGraw et al. 2002), which could explain the lack of relationship between condition and other structural plumage patches in the present study. Alternatively, blue tits could obtain information about the quality of conspecifics from traits other than ornaments (i.e. song repertoire, Doutrelant et al. 2000), which may add or interact with information provided by feather colouration. Although we do not present data on singing activities in the blue tit, our results showed marginally non-significant associations between colour in different patches and infections by some haemoparasites (see Tables 1 and 2). However, we cannot exclude the possibility that the observed associations respond to complex interactions between immune system

responses and feather synthesis during the moult (Sanz et al. 2004; Serra et al. 2007; Orledge et al. 2012), so marginal results should be taken carefully. The challenge in future studies will be to distinguish empirically whether different ornaments are redundant or non-redundant by exploring the behaviour they elicit from a recipient (Partan and Marler 1999).

Another noteworthy point is that a single ornament may provide two pieces of information via different colour characteristics. Senar et al. (2008) study supports this hypothesis, but in a carotenoid patch (via hue and chroma) in the closely related great tit *Parus major*. In this study, we found a similar pattern in the white cheek (via brightness and saturation). Male blue tits that grew more saturated white cheeks were more intensely infected by *Plasmodium* in the spring of season 1. It is possible that more saturation in white patches signals poorer individual quality (Badás et al, 2017). This result is, at least partly, in accordance with previous findings in the present blue tit population. In another study carried out in the 2012 breeding season (Badás et al 2017), we found that males infected with a different haemosporidian parasite species, *Haemoproteus*, had more saturated white cheeks, and in turn these males paired with lower quality females. Two hypotheses can be proposed to explain why different parasites species were found to affect cheek colouration: (i) parasites could increase their level of virulence depending on environmental conditions (Møller et al. 2013), or (ii) infections by *Plasmodium* could be positively correlated with infections by another undetected parasite that disrupts feather structure in the white cheek. For example, an experimental study that inoculated wild turkeys (*Meleagris gallopavo*) with coccidial oocysts found reduced UV reflectance in a structural plumage patch (Hill et al. 2005). Although speculative at the moment, *Plasmodium*-infected individuals could also be infected by coccidians such as *Isospora* sp., which have been found to infect blue tits in our population (del Cerro, S., unpublished data). Besides, multiple infections with parasites other than haemosporidians are common in this blue tit population (Merino et al. 1997; del Cerro et al. 2010).

We also found that male blue tits with brighter cheeks may have been able to attract better quality females in the following spring (season 2), because the male's partners in season 2 were brighter than those males' partners in season 1. Unfortunately, we were unable to explore breeding success as a result of matting with higher ornamented females in season 2 because a post-hatching experiment was taking place in the spring of 2014. Still, we present, for the first time, data on change in feather colour after the moult, which is associated to mating patterns in the consecutive season. This relationship is further supported by a discrimination model that takes into account the birds' visual system. Additionally, it should be noted that high quality individuals might not change colour between seasons in a conspicuous way (as shown by the fact that most individuals had low JND scores between seasons, Table A2 in the Appendix). Confirmation for this hypothesis was found in our results: male blue tits with brighter cheeks in season 1 changed less between seasons. The reason why individuals may not change cheek brightness in a conspicuous way may be due to genetic effects, which could affect colour expression in the structural white in blue tits. Variation in the yellow breast and blue crown colour was found to be weakly heritable in adult blue tits (Hadfield et al. 2006), whereas in nestlings, reflectance in another structural plumage patch, the blue-green tail, has been suggested to be influenced by paternally inherited genes (Johnsen et al. 2003). The possibility of genetic effects in white colour expression remains unexplored in this species.

By using the latest methods of analysis of reflectance, our results, although correlational, suggest that better performance during the reproductive season (i.e. regarded as higher body mass and/or less intense infections by blood parasites), may have important implications for the following breeding event. Blue tit males that were in better body condition at the highly demanding nestling provisioning stage were able to develop brighter white cheek feathers after the moult. This might have enabled them to find brighter females than those they paired with in the previous spring. We also offered the

first correlational evidence that intense infections by *Plasmodium* during a costly reproductive stage might have consequences for the moult. A visual discrimination model confirmed that differences in colour could be perceived by conspecifics. This study sets the basis for further experimental studies on the carry-over effects of reproduction in ornamentation (but see Doutrelant et al. 2012) and mating patterns. Allocating resources efficiently during reproduction to immune defence and self-maintenance may increase reproductive success in the following reproductive period.

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APPENDIX

Supplementary tables

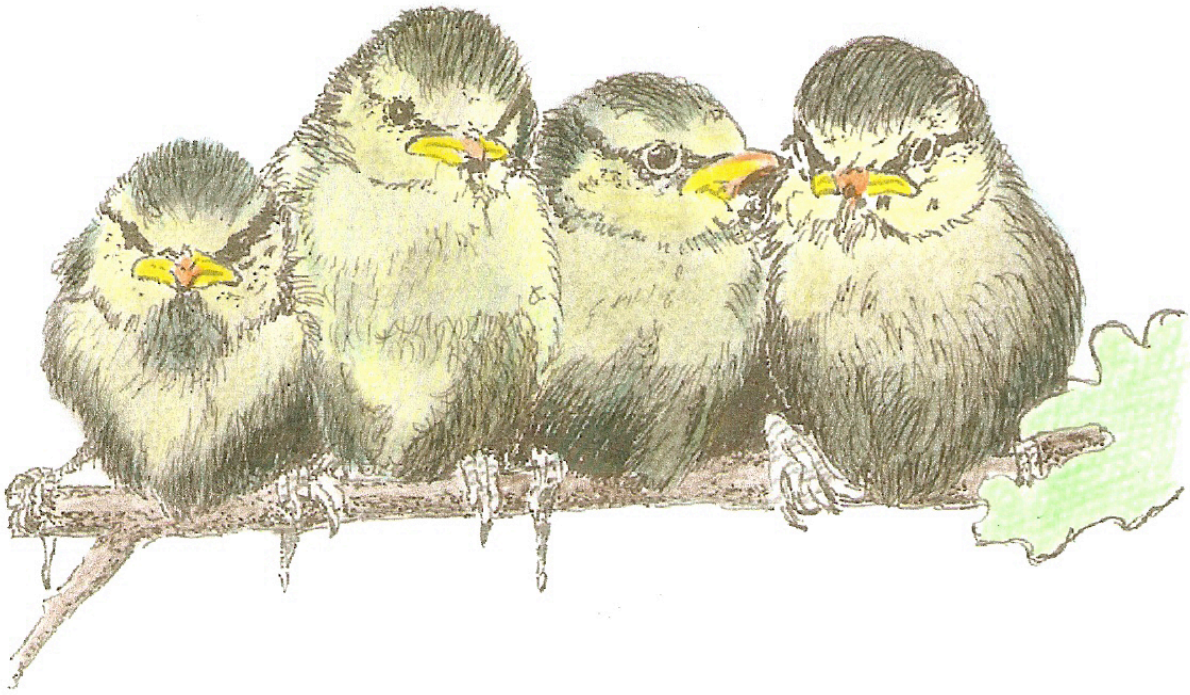
Table A1. Models (within ΔAICc < 10 units) explaining colour change in blue tits using the Akaike's second-order Information Criterion (AICc). Variables included in each model were marked with "X". A total of 30 males were included in this analysis.

Model		Predictors						AICc	ΔAICc	AICw
		Sample	Body Mass	*Haemoproteus*	*Plasmodium*	*Leucocytozoon A*	*Leucocytozoon B*			
Cheek saturation	1	X			X			-116.44	0	0.52
	2	X	X					-114.73	1.71	0.22
	3	X					X	-113.31	3.13	0.12
	4	X				X		-112.82	3.62	0.09
	5	X		X				-112.08	4.36	0.06
Cheek brightness	1	X	X					-33.1	0	0.85
	2	X				X		-29.06	4.04	0.07
	3	X					X	-28.27	4.83	0.05
Breast saturation	1	X	X					-107.08	0	0.36
	2	X				X		-107.02	0.06	0.35
	3	X		X				-104.71	2.37	0.11
	4	X					X	-104.42	2.66	0.10
	5	X			X			-104.09	2.99	0.08
Tail brightness	1	X					X	-121.41	0	0.85
	2	X		X				-121.35	0.06	0.07
Crown saturation	1	X	X				X	-77.91	0	0.65
	2	X				X		-75.68	2.23	0.21

Table A2. Chromatic and achromatic colour contrasts describing differences in male feather colour. The differences were calculated by comparing feather colour for each individual between the spring vs. winter of 2013, and the winter of 2013 vs. the 2014 breeding season. In order to be conservative, the threshold used for JNDs was set at 5 scores. A higher number of individuals below threshold may suggest that the change between colours was not perceptible from an avian receiver.

		Spring 2013- Winter 2013				Winter 2013- Spring 2014			
Patch	**Type of colour contrast**	**JND mean ± SE**	**% of individuals below threshold**	**Maximum JND**	**N**	**JND mean ± SE**	**% of individuals below threshold**	**Maximum JND**	**N**
Cheek	Colour	3.25±0.31	86%	6.55	22	3.31±1.1	82%	5.18	12
	Luminance	7.89±1.31	38%	24.69	22	5.92±3.06	67%	12.41	12
Breast	Colour	4.69±0.46	38%	10.26	22	3.55±0.64	92%	5.14	12
	Luminance	3.83±0.62	71%	10.72	22	4.44±3.15	58%	8.99	12
Tail	Colour	4.87±0.79	60%	14.54	21	3.79±1.22	73%	5.76	11
	Luminance	6.05±0.96	50%	15.36	21	6.62±3.33	27%	13.59	11
Crown	Colour	3.95±0.46	81%	10.66	22	2.3±1.51	92%	5.89	12
	Luminance	5.68±3.5	48%	13.12	22	6.43±4.71	50%	15.24	12

Chapter 2



'The years of early childhood are the time to prepare the soil.'

—Rachel Carson

This chapter reproduces entirely the manuscript:

E.P. Badás, J. Martínez, J. Rivero-de Aguilar, and S. Merino. Experimental reduction of parasites in early-life affects colour expression in blue tits.

Experimental reduction of parasites in early-life affects colour expression in blue tits

E.P. BADÁS, J. MARTÍNEZ, J. RIVERO-DE AGUILAR, AND S. MERINO

Abstract Cavity-nesting birds are exposed to haematophagous ectoparasites and blood parasitic infections already at the nestling stage. Although there is good evidence that ectoparasitic infections have a negative impact on nestlings, the combined effect of multiple parasite species on developing birds is unknown. Moreover, their effect on feather colouration is unclear. In a blue tit (*Cyanistes caeruleus*) population in central Spain, during the spring of 2013, we administered the following treatments aimed at reducing the parasite community infecting nestlings: a group of nests was sprayed with an insecticide, while, in other group, nestlings were administered with anti-malarial medication. Before fledging, feather colouration in the carotenoid-based yellow breast and the structural blue-green tail was measured. Additionally, we detected or quantified blood parasites and nest ectoparasites. The avian malaria-like parasite *Leucocytozoon* spp. was successfully reduced in medicated nestlings. In insecticide-sprayed nests, blood-sucking flying insects and nest-dwelling ectoparasites' populations were reduced, and nestlings were less likely to be infected by *Leucocytozoon* spp. in both sexes, and *Trypanosoma* spp. in males. We found no effect of the treatment in yellow plumage colouration. However, nestlings from insecticide-sprayed nests had less saturated tail feathers, and tended to have higher body mass. In winter, yearlings that had been reared in sprayed nests grew brighter blue crown. We provide the first experimental evidence that parasite reduction during the nestling stage could have an effect on sexual ornaments, which may have important consequences for the bird's first breeding attempt. The efficacy of the treatment and the potential negative effects of parasites on infections acquired in early life and are also discussed.

Keywords body mass, haematozoans, prevalence, sexually-selected signals, virulence

INTRODUCTION

In birds, individuals are confined to the nest during development, where they are exposed to several pathogens (Rivero-De Aguilar et al. 2016). For example, it is now generally accepted that infections by nest ectoparasites impose a challenge to developing nestlings, for example, by decreasing body condition and fledgling success (Martínez–de la Puente et al. 2010; Wegmann et al. 2015), increasing stress proteins levels (Merino et al. 1998), or reducing anti-oxidant defences (López-Arrabé et al. 2015). Surprisingly, the costs of ectoparasitic infections in nestling feather colouration are less clear. Carotenoid-based colouration depends on acquiring carotenoids from the diet and then depositing them to feathers (Hill et al. 2002), and it has been described as an honest signal of immunocompetence and health status (Saks et al. 2003). Nestlings reared by higher quality parents should grow more ornamented yellow plumage patches, since foraging abilities are related to carotenoid-based colouration (García-Navas et al. 2012); and/or they may be able to cope with ectoparasitic infections thanks to the immune activation provided by carotenoids (Saino et al. 1999). Contrary to this hypothesis, in adults from the closely related great tit (*Parus major*), yellow breast colouration was not affected by flea-infestations, while other ornaments increased their quality in uninfected nests (i.e. the size of the melanin-based breast stripes Fitze & Richner, 2002). This is unexpected, especially because it has been reported in another passerine, the blue tit (*Cyanistes caeruleus*), that nestlings increase begging when they are reared in nests with high flea infestations; and their parents compensated the negative effects of such parasitic infections with higher food provisioning (i.e. carotenoids) (Tripet and Richner 1997). However, because nests are infected with several species of nest parasites (Rivero-De Aguilar et al. 2016), other infections may negatively affect carotenoid deposition or ornamental feather colouration. The complex interactions between multiple ectoparasites on feather colour are, thus, understudied.

Haematozoan parasites also infect passerines during the breeding season (Valkiūnas 2005). The most common genera include the well-known avian malaria parasite *Plasmodium* spp., or other haemosporidians referred to as malaria-like parasites, mainly *Haemoproteus* spp. and *Leucocytozoon* spp. (Atkinson and van Riper 1991; Pérez-Tris et al. 2005). Because spring is the period of maximum vector abundance in temperate regions (Pérez-Rodríguez et al. 2015), the transmission of vector-borne parasites occurs when reproductive adults and immunologically naïve individuals like nestlings are more susceptible (Dowell 2001). In adult birds, relapses from chronic infections are frequent (Valkiūnas 2005); but nestlings are faced with their first encounter with the parasite at the nest. In fact, the initial phase of acute parasitaemia when first exposed to the parasite is known to be the most dangerous and even lethal stage of malarial infections in some bird species (Palinauskas et al. 2009; Lachish et al. 2011).

Some studies have related infections by *Leucocytozoon* spp. to higher stress levels in nestling blue tits (Arriero et al. 2008). Infections by another protozoan, *Trypanosoma* spp. may have negative effects in the nestling's immune system in blue tits (Martínez-de La Puente et al. 2013), and in stress levels in house martins (*Delichon urbica*) (Merino et al. 1998). Parasites such as *Haemoproteus* spp. or *Plasmodium* spp., on the contrary, are rarely found in nestlings (Cosgrove et al. 2006). In fact, the negative effects of blood parasitic infections in developing birds may be underestimated, because most parasites are not detected in circulating blood until after 5 to 15 or more days (Fallis and Bennett 1961; Cosgrove et al. 2006), and few studies have sampled nestlings before the prepatent period (Martínez-de La Puente et al. 2013).

In the avian malaria system, coinfections between several haematozoans are abundant (Muturi et al. 2007; Rooyen et al. 2013; Clark et al. 2016), and pathogen interactions may increase the host's susceptibility to additional infections (Marzal et al. 2008). In developing nestlings, these interactions may even be more harmful than in

adults, because the investment in immune responses is tightly controlled by trade-offs between immunocompetence and growth (Brommer 2004). In fact, only hosts in good condition might be able to mount costly immune responses against parasitic infections (Tschirren et al. 2007; Cornet et al. 2014). Therefore, nestlings infected with both ectoparasites and blood parasites are likely to suffer from an impaired immune system and may have fewer resources (i.e. carotenoids) to allocate to feather colouration and/or growth. Little is known about the interplay between parasites, growth and ornamental colouration in nestling birds.

In this study, we explored the consequences of experimentally reducing ectoparasites load and/or infections by haematozoans in the blue tit *Cyanistes caeruleus*. In order to do so, a group of blue tit nests was sprayed with an insecticide, another group of nestlings was administered with an anti-malarial drug, and a final group of birds acted as control. With the insecticide spray, we expect to significantly reduce ectoparasite populations infecting blue tits' nests. Indirectly, we also expect to reduce infections by haematozoans by targeting vectors that may transmit infectious diseases such as the ones described above. In the medicated group, blood parasites were targeted directly with the medication. Molecular data from the preceding season in this study population showed that the prevalence of blood parasites in blue tit nestlings is low (1.3% for *Haemoproteus*, 3.9% for *Leucocytozoon* haplotype A, 9.1% for *Leucocytozoon* haplotype B and 4.4% for *Trypanosoma* spp. in the 2012 breeding season, N=640, data not published). Still, the use of the anti-malarial drug in this study was prophylactic. We expect to find lower prevalence of infections in both treatment groups with respect to the control group. In fact, this experimental design may allow us to investigate whether the reduction of blood parasites caused by medication or the reduction of both blood parasites and ectoparasites by the insecticide had a differential effect on feather colouration, fitness parameters or nestling growth.

We explored the effect of the treatment on feather colouration in two plumage patches in blue tit nestlings: the yellow breast and the blue-green tail. As explained above, parasitic infections may exert negative effects on pigment deposition (Hill 2006), and in turn, they could affect carotenoid-based colouration. Thus, we expect to observe increased saturation and brightness in plumage colouration in nestlings from both experimental groups when compared to the control group. In this species the blue-green tail colouration results from a yellow pigment component and a blue structural component (Hill 2006), and it is sexually dimorphic in nestlings (Peters et al. 2007). Blood parasites have been found to negatively affect structural iridescent colouration in the satin bowerbirds, *Ptilonorhynchus violaceus* (Doucet and Montgomerie 2003); but to our knowledge, no study has yet explored the relationship between parasitic infections and structural colouration in nestling birds.

The conditions experienced during early development may have long-term effects on other parameters indicating individual performance (Lindström 1999). For example, ectoparasite infestations during the nestling phase induce costs on survival and lifetime reproductive success in great tits (Fitze et al. 2004). In blue tits, nestling body condition explained resistance to infections by malarial parasites one year later (Stjernman L.; Nilsson, J.-Å. et al. 2008). And in the hihi (*Notymystis cincta*) improved nutritional conditions in early life increased saturation in a carotenoid-based ornament after moulting (Walker et al. 2013). Thus, we also expect to find an effect of the treatment on feather colouration in the long-term, and we tested this by recapturing some individuals in winter. In nestlings that remained uninfected during development, we expect increased colouration after the post-juvenile moult in ornamental patches.

We also explored whether extra-pair paternity explained susceptibility to infections by blood parasites, because genetic effects may explain resistance or susceptibility to malarial parasites (Ferrer et al. 2014). In fact, previous studies in this blue

tit population have reported the presence of extra-pair nestlings in nearly half of the nests (Badás et al. 2017-**Chapter 3**).

Finally, as a result of reducing several species of parasites, we expect to find higher fledgling success and nestling body mass in treated nestlings.

METHODS

Study population

During the spring of 2013 we studied a population of blue tits *C. caeruleus* breeding in nest-boxes in a Pyrenean oak *Quercus pyrenaica* forest located in Central Spain (Valsaín, 40º53'74 N, 4º01' W, 1200 m.a.s.l.). Periodical visits in the course of the breeding season allowed us to record hatching date, brood size, nestling growth and identity of the parents at each nest. Nestlings were marked individually at day 3 (hatching=day 0) using nail paint and weighed to the nearest 0.1g at day 3, 9, 10 and 15. The weights on day 15 were denoted as fledging weight. Tarsus was also measured to the nearest 0.01mm each time the nest was visited, and on day 15 nestlings were also ringed and a drop of blood was obtained from the brachial vein. Mass was corrected by regression for body size (tarsus length) and time of day (Senar 2002). Adult birds were caught using an automated nestbox trap while feeding their young (day 3), ringed if necessary and weighed. During the winter of 2013 (November and December) some nestlings were recaptured as first-year adults using song-baited mist nets (see **Chapter 1**).

Experimental procedure

At the beginning of the breeding season blue tit nests that shared similar hatching date (±1 day) and clutch size (±1 egg) were randomly assigned to one of three treatments: medicated, insecticide-treated and control. Because the aim of this study was to reduce avian malarial infections in nestlings by using insecticide or prophylactic methods, we were not interested in a full-factorial experimental design. Thus, in order to avoid further

reductions in sample size, the group insecticide-sprayed + medicated was removed. Instead, we used an incomplete factorial design with three levels: water-sprayed + water-administered (control group), water-sprayed + medicated (medicated group hereafter), and insecticide-sprayed + water-administered (insecticide-treated group). Nestlings from the medicated group of nests were administered with an antimalarial drug dissolved in water orally to the bill (7 µg/g following Knowles *et al.*, 2010). Medication was administered at nestling age 3, 9 and 10. We chose Malarone™ (Atovaquone and Proguanil Hydrochloride; GlaxoSmithKline, Brendtford, UK) because it is a highly effective anti-malarial drug with few side-effects in humans (Looareesuwan et al. 1999). Besides, complete clearance of *Plasmodium* infections from blood after the use of this drug has been observed in several passerine species (Palinauskas et al. 2009), including blue tits (Knowles et al. 2010). Nestlings from the control and insecticide-treated nests were administered orally with the same amount of water. Nests from the insecticide group were sprayed on the inside and outside walls of the nestbox with Stockade© (Fort Dodge Veterinaria, S.A., Vall de Bianya, Girona, Spain) at several occasions in order to ensure the efficacy of the insecticide: once during incubation (2 days before the expected hatching date), and three times during chick development (at nestling age 3, 9 and 10 days old). The insecticide solution contained 0.5% Permethrin and 1% Piperonyl butoxide, and it has been shown to effectively reduce ectoparasite load in blue tits nests in the present population with no harmful effects to developing nestlings (Tomás et al. 2007; Martínez–de la Puente et al. 2009). At each spraying event, either the clutch or the brood was removed from the nestbox, and it was carefully placed back after the administration of the chemical compound. Nests from the control and medicated groups were visited on the same occasions and sprayed with water on the inside and outside walls of the nestbox.

Colour measurements and models of colour vision

Colour reflectance data on nestlings were collected on day 15 using a portable spectrophotometer (Ocean Optics Inc., Dunedin, FL, USA) in combination with a xenon lamp (PX-2; Ocean Optics). These were connected via a bifurcated fibre-optic probe, at the end of which a cylindrical probe-holder was fitted in order to ensure that measurements were taken at a uniform distance from the feather surface (5.0 mm) and that ambient light was excluded. Measurements were taken at two different feather patches: the yellow breast and the blue-green feathers at the base of the tail. Reflectance was assessed relative to a dark standard and a white Spectralon tile. Three measurements were taken for each patch and the mean of these three was used as the plumage reflectance measure for that sample. In order to determine how these colours are perceived by birds we used models of colour vision that are based on the known photoreceptor spectral sensitivities for the blue tit (Hart et al. 2000), and thus extracted the relative photon catches for the single and double cones used in bird vision (Endler and Mielke 2005; Stevens et al. 2009). Thus, three colour variables were calculated: hue, brightness and saturation. The use of these variables has recently provided important insights into ecological and evolutionary aspects of animal visual communication (for a detailed description on how these variables were obtained, see **General Methods Chapter**).

Parasite quantification

From day 10 until day 13 of nestling age a Petri dish that contained gel oil was attached to the roof of each nestbox following Tomás *et al.* (2008). Thus, blood-sucking flies from the Ceratopogonidae family (biting midges, *Culicoides* spp., Martínez–de la Puente *et al.*, 2009) and blackflies from the Simuliidae family were sampled inside the nestbox during the nestling-rearing period. The ectoparasites' abundance at each nest was estimated by counting the insects that were glued to the Petri dish under the binocular lens (Olympus SZX7). This method has successfully been used to quantify blood-sucking

flying insect loads in this blue tit population (Tomás et al. 2012; Rivero-De Aguilar et al. 2016).

After all nestlings had fledged (20 days post-hatching), wholes nests were removed from the nestbox and stored at 4ºC until examined. Nest-dwelling ectoparasites such as mites (*Dermanyssus gallinoides*) or flea larvae (*Ceratophyllus gallinae*) were collected in vials containing 70% ethanol after a defaunation procedure in Berlese funnels for 48 hours and later quantified under the binocular lens (see Merino & Potti, 1995). Other blood sucking ectoparasites such as larvae from the blowfly (*Protocalliphora azurea*) were counted when dismantling the nest material.

For the molecular detection of intracelullar parasites like *Trypanosoma* spp. and the malaria-like *Leucocytozoon* (haplotypes Leu A and Leu B, see **General Methods Chapter**), DNA was extracted using a standard ammonium-acetate protocol from blood samples collected when nestlings were 16 days old. Infections by *Haemoproteus* and *Plasmodium* parasites were not explored because previous data in this population revealed very low prevalences of infection (see above). *Leucocytozoon* parasites were detected through a PCR that amplifies a fragment of the cytochrome B region of the parasite (using the species-specific primers described in the **General Methods Chapter**). *Trypanosoma* spp. was detected using the same PCR method and a pair of species-specific primers (forward primer, TryF: 'ATGCACTAGGCACCGTCG' and reverse primer, TryR: 'GGAGAGGGAGCCTGAGAAATA'; GenBank accession number KJ415280). Polymerase chain reactions (PCRs) were carried out in 10 L reaction volumes. The reactions were cycled under the following conditions: annealing at 60º for 30 seconds followed by an extension at 60º during 30 seconds.

Paternity analyses and sexing

Parents and nestlings were genotyped for 8 microsatellite loci; information on microsatellites, primers and polymerase chain reaction (PCR) conditions are detailed in the **General Methods Chapter**. Fluorescently labelled PCR products were separated on an AB3730 DNA analyser. Allele lengths were determined with the Genemapper 4.0 software. The offspring was assigned as extra-pair if there were at least two mismatches between the genotype of the social father and offspring. Maternity of the social female was confirmed for all nestlings. The mean exclusion probability of the eight markers was calculated to be 0.99968 for the first (female) parent and 0.99999 for the second (male) parent (given the genotype of the first parent).

Nestlings were sexed using the amplification by PCR of the chromo-helicase-DNA-binding (CHD) genes based on Griffiths *et al.*, (1998). In birds, these genes are located on W and Z chromosomes. The set of primers (P1 and P8) amplifies homologous sections of both genes and incorporates introns whose lengths usually differ. Therefore, females (ZW) render two bands and males (ZZ) only one. PCR were performed in a 10 µL reaction volume containing between 20–100 ng template DNA, 0.25 µM of each primer, and Supreme NZYTaq 2x Green Master Mix (NZYTech, Lda. - Genes and Enzymes, Portugal). PCR conditions are as follows: 95 °C for 10 min (polymerase activation), 40 cycles at 95 °C for 30 s, annealing temperature 45 °C for 30 s, extension temperature 72 °C for 30 s, and a final extension at 72 °C for 10 min. PCR assays were checked using agarose gel (1.5%) electrophoresis.

Statistical analyses

All analyses were performed in R v.3.1.3 (R Foundation for Statistical Computing, Vienna). Overall, 79 nests were included in the experiment: 27 medicated, 30 insecticide-sprayed and 22 control nests. Data from 15 nests could not be obtained because of nest desertion, which was marginally dependent of the treatment group (Fisher's Exact Test, p-

value= 0.05329). However, the occurrence of nest desertion was probably due to extreme weather conditions during the 2013 breeding season (as seen in other blue tit populations throughout Europe, Glądalski *et al.*, 2014) and not to a harmful effect of the anti-malarial drug or the insecticide, because 9 out of 15 deserted nests belonged to the control group of nests. Sample sizes differ slightly between analyses because some measurements could not be obtained for all nests (i.e. lack of blood for parasite quantification or paternity analyses, spectrophotometer failure).

In order to test the efficiency of the treatment in the elimination of several parasite species infecting blue tits nests, we designed linear or mixed linear models depending on the nature of the independent variable. First, for ectoparasite counts on each nest, we used generalized linear models (GLMs) with negative binomial error distribution or hurdle negative binomial models to handle an excess of zeros in count data. Hatching date, brood size on day 15, mean nestling body mass on day 15 and treatment were included as dependent variables. Model residuals were checked for normality and homoscedasticity. Because we found influential points for some models, robust models based on the Cook's distance and leverage (Montgomery et al. 2012) were built but gave similar results. Second, to examine the effect of the treatment on the presence/absence of intracellular parasites in nestlings (N=380), we used general linear mixed models with binomial error distribution (or and ordinal model using the R package 'ordinal', see the Results section) and nest identity as random factor. Separate models were fitted for each parasite species and variables included in the best-fitting model were chosen on the basis of the AIC (Akaike Information Criterion, Sugiura, 1978) and model averaging using the R package 'glmulti' and weights over 0.5 following Galipaud *et al.* (2014). Variables in the saturated model included: treatment, sex, hatching date, relative growth, wing growth, nestling's body mass on day 15, brood size and the interaction between treatment and sex. We weighed all nestlings on all three visits to estimate relative growth as the percentage of weight gained during treatment divided by nestling weight at the first visit (Soler et al.

2003). We also calculated relative growth until nestling day 10 but this variable was not included in the models because it was highly correlated to relative growth until nestling day 15 (Pearson's correlation: t = 63.19, df = 363, p-value < 0.0001, r=0.957). Wing growth was estimated in a similar way using wing length at the beginning of the treatment and wing length on day 15. Nestlings that were infected by *Leucocytozoon* A were more likely to be infected by *Leucocytozoon* B (Exact test, Z=10.495, p-value<0.0001), and thus this haplotype was included in the model when testing for an effect of the treatment in *Leucocytozoon* A (and vice versa). Significance against the null model was tested using LR tests and p-values for the fixed effects were obtained using the Bayesian implementation in the R package 'MCMCglmm' (Hadfield 2010). Univariate models were run for 250000 iterations per model, from which we discarded the initial 3000 (burn in period). Each chain was sampled at an interval of 250 iterations, which resulted in a low autocorrelation among thinned samples (<0.05). Posterior means and 95% credible intervals were estimated across the thinned samples for the mean effects (fixed effects) and (correlations). The results presented here correspond to parameter extended priors, which can improve mixing considerably for variances that are close to zero (Hadfield 2010). In addition to this, we explored whether extra-pair or social nestlings were more likely to be infected by intracellular parasites using unconditional Boschloo Exact tests for unbalanced samples (Boschloo 1970). Extra-pair paternity could not be included in the mixed effect models as an explanatory variable because convergence was not reached (due to the reduced sample size of extra-pair nestlings across treatments and nests, N=96 total extra-pair nestlings, see Results section).

The effect of the treatment on the nestlings' feather colouration (N=358) was investigated using a multivariate analysis of variance (MANOVA) with hatching date, relative growth, wing growth, nestling's body mass and brood size on day 15 as dependent variables. The interaction between treatment and sex was also included. We fitted separate models for each colour variable and patch (yellow saturation, yellow brightness,

green saturation and green brightness). Hue was not tested because it was highly correlated to saturation in both feather patches (Pearson's correlation breast: t = 20.37, df = 534, p-value < 0.0001, r=0.66, tail: t = -9.22, df = 530, p-value < 0.0001, r=0.37), and saturation is commonly chosen over hue (Badás et al. 2017-**Chapter 3**). Nest identity was incorporated in all models as random factor to avoid pseudoreplication. Estimates of significance for the main effects were calculated from 1,000 bootstrapped iterations derived with 'bootMer' from the R package lme4 (Bates et al. 2014). The colour variable 'tail saturation' was inverse-transformed in order to meet model assumptions of normality. In this model, we found two outliers during residuals checking but similar results were obtained when this data points were removed from the analyses. Thus, data presented here include all data points.

We also investigated discriminability of colour change and colour variables in a subsample of 15 nestlings for which data on winter recapture was available. Colour change was tested on plumage patches that are already present at the nestling stage (yellow breast and blue-green base of the tail) and maintained during the first reproductive event, because feather colouration because could fade during the season due to bacterial degradation (Shawkey et al. 2007), or feather wear, which could be faster in young birds (Ferns and Hinsley 2004). In order to do this we used chromatic and achromatic just-noticeable differences (JND scores, see Badás et al. 2017, **Chapter 3** for more details). Other plumage patches such as the white cheek and the blue crown are developed after the post-juvenile moult, and therefore they could only be measured in first-year adults captured in winter. In order to explore the effect of the treatment in colour change or feather colouration in all these patches, we used pairwise comparison with Welch two-sample t-test because it is more reliable when the two samples have unequal sample sizes ($N_{control}$= 3, $N_{malarone}$= 7, N_{spray}= 5) (Ruxton 2006). Effect sizes were calculated by means of Hedge's G, an alternative for Cohen's D that provides a measure of effect size weighted to the relative size of each sample (Cohen 1998).

Finally, we used a generalized linear model (GLM) with binomial error to evaluate the effect of the treatment on fledgling success. Fledgling success was computed as the proportion of hatched young that reached 15 days of age (Badás et al. 2015). In order to examine the effect of the treatment in nestling body mass we designed two different models. First, we used a linear mixed effects model with nest identity as random factor and hatching date, brood size, relative growth, wing growth, treatment, sex, and the interaction between treatment and sex as dependent variables. Second, following Martínez–de la Puente *et al.*, (2010) we tested the interaction between abundance of biting midges and presence/absence of black flies on nestling mass. In order to do this, we used mean nestling body mass per nest and designed a linear model with hatching date, brood size, abundance of mites, blowfly larvae and biting midges, and the interaction between biting midges and presence/absence of black flies. The final model was selected on the basis of AIC and final p-values for the main effects were obtained from a robust regression based on leverage and re-weighted least squares using the R package 'wle', which reduces the effect of outliers and influential points (Crawley 2013).

RESULTS

Effect of the treatment on nest ectoparasites and haematozoans

The abundance of several species of ectoparasites differed across treatments (Table 1). Nests sprayed with insecticide showed significantly less flying blood-sucking (biting midges: Z-value=-3.03, p-value=0.00245; blackflies: Z-value=-2.82, p-value=0.00481) and nest-dwelling parasites (blowfly larvae: Z-value=-3.16, p-value=0.002459; mites: Z-value=-4.01, p-value<0.0001;) with respect to control and medicated nests (Fig. 1). Additionally, biting midges and nest-dwelling ectoparasites were affected by phenology estimated as hatching date: early broods were less infected by parasites (Table 1). The abundance of blackflies in blue tits' nests increased significantly

with the number of nestlings (Table 1). The effect of the treatment on flea larvae was not tested because they were only found in 5 nests (N=79).

Table 1. Generalised linear models for the ectoparasite counts infecting blue tits' nests. Model AIC refers to the AIC value of the full model and AIC refers to the value of the model without the selected predictor. Significant p-values are highlighted in bold.

Parasite count	Predictor variable	χ^2	P-value	Model AIC	AIC	R^2 (%)	Prevalence (%)	Sample size
Culicoides spp.	**Treatment**	8.17	**0.01686**	359.53	363.70	41.93	63.89	68
	Hatching date	24.72	**0.000007**		382.25			
	Mean nestlings' body mass	0.49	0.48		358.03			
	Brood size	1.26	0.26		358.79			
Simuliidae	**Treatment**	20.92	**0.000029**	326.08	343.00	30.71	76.39	68
	Hatching date	3.13	0.07687.		327.21			
	Mean nestlings' body mass	0.93	0.34		325.01			
	Brood size	4.15	**0.04161**		328.24			
Protocalliphora larvae	**Treatment**	21.35	**0.00002312**	348.01	362.74	13.02	40.51	78
	Hatching date	8.41	**0.003732**		348.50			
	Mean nestlings' body mass	0.58	0.45		344.90			
	Brood size	1.77	0.18		346.75			
Mites	**Treatment**	20.12	**0.000043**	505.72	521.85	27.45	69.62	78
	Hatching date	8.096	**0.0048**		511.82			
	Mean nestlings' body mass	1.88	0.0668.		505.60			
	Brood size	0.56	0.24		504.28			

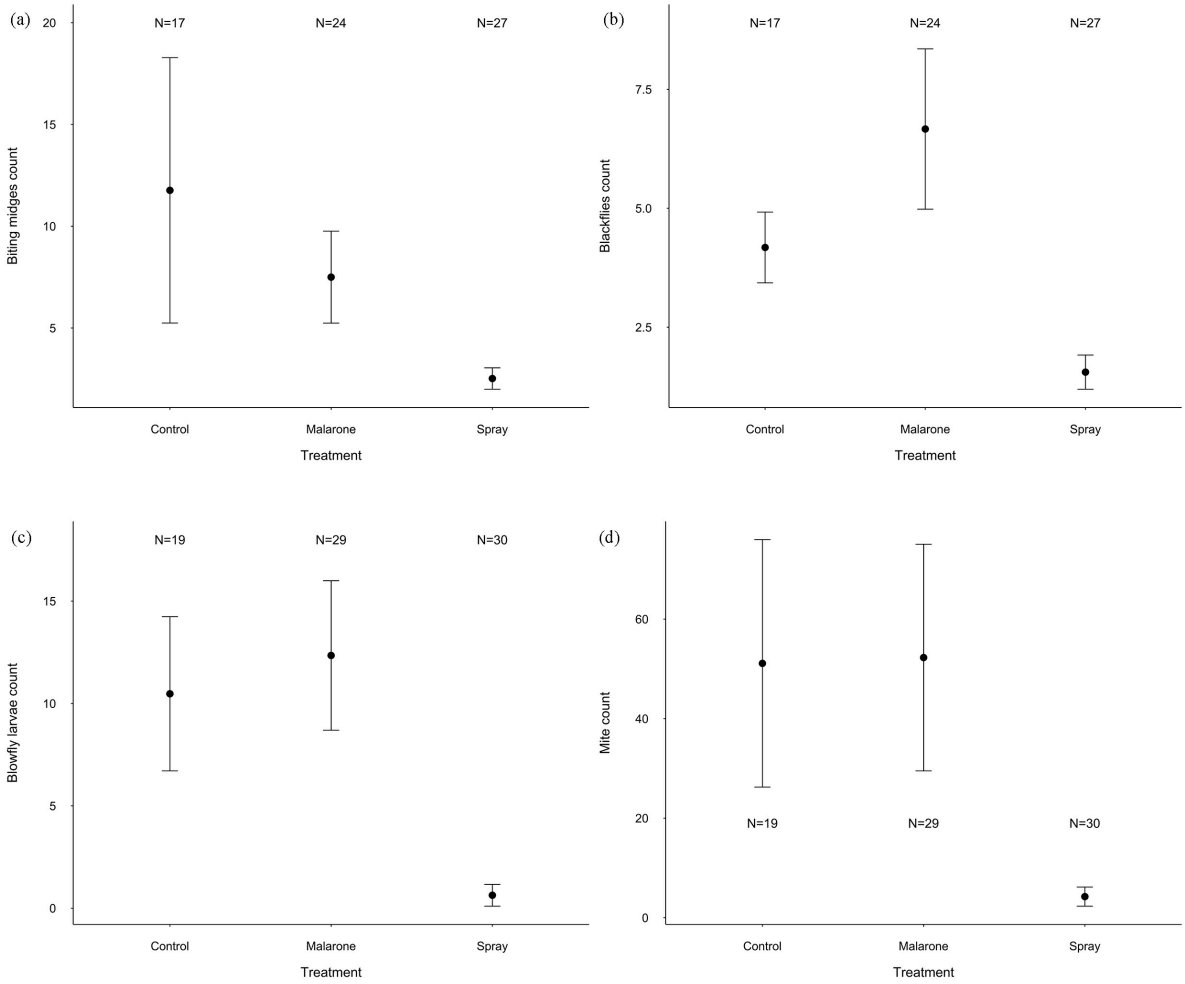


Figure 1. Effect of the treatment on ectoparasite counts: (a) *Culicoides* spp., (b) Simuliidae, (c) *Protocalliphora azurea* larvae, (d) *Dermanyssus gallinoides*. Sample sizes for each treatment group are shown above bars.

Infections by blood parasites were also different across treatment groups. The odds of being infected by *Leucocytozoon* A were significantly lower in nestlings from the medicated and insecticide-sprayed nests (Table 2, Fig. 2) and in nests that hatched later in the season (Table 2, Fig. 2); and higher in nests with larger brood size (Table 2, Fig. 2). An ordinal model that tested the odds of being infected by none, one or both *Leucocytozoon* haplotypes confirmed that the odds of remaining uninfected were higher in treated nests (Table 2, Fig. 3). However, the odds of being infected by *Leucocytozoon* B were not different across treatment groups when it was tested separately (Table 2). Finally, male nestlings from the insecticide-sprayed group were also less likely to be infected by *Trypanosoma* spp., as seen by a significant interaction between treatment and sex (Table 2, Fig. 4).

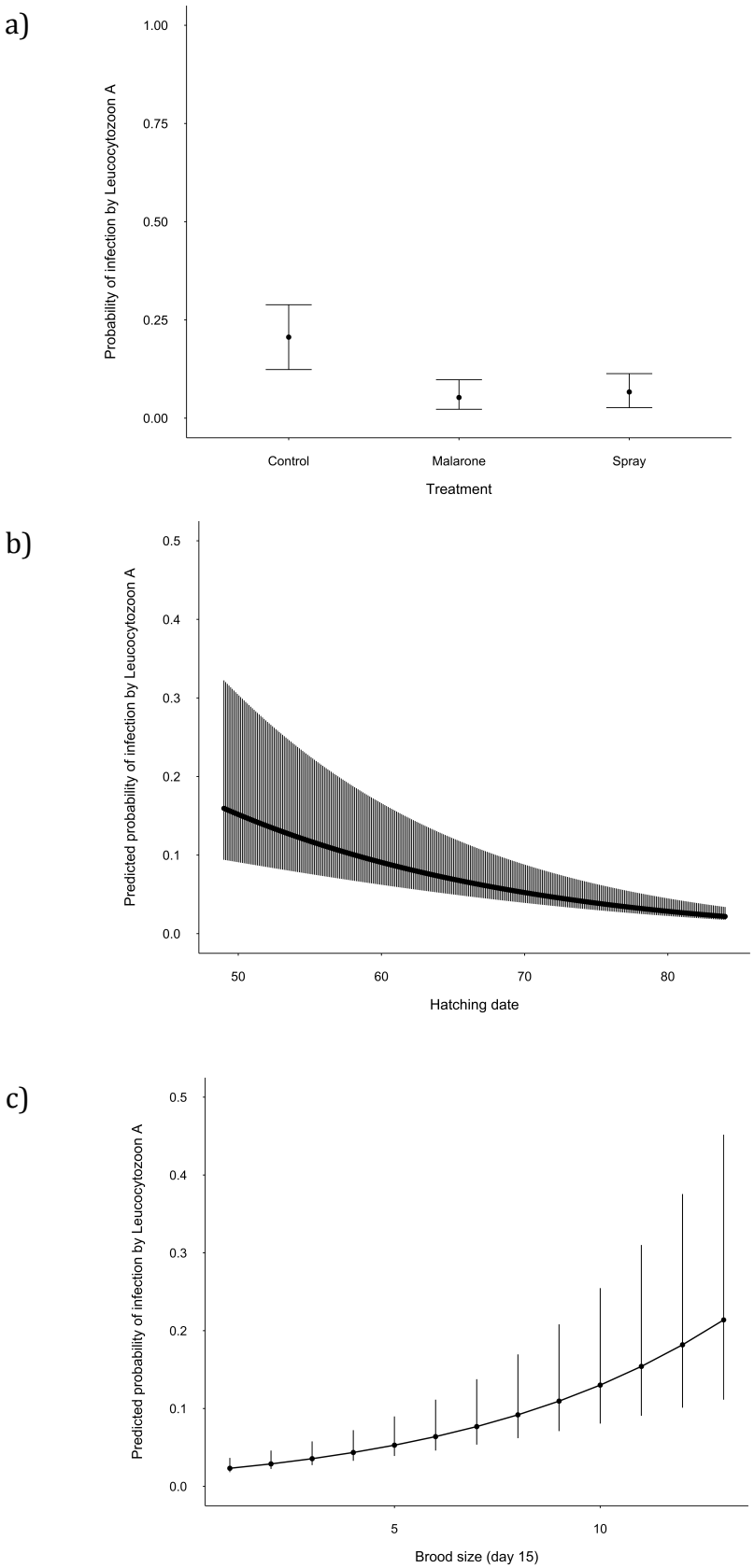


Figure 2. Nestling infection by the blood parasite *Leucocytozoon* A. (a) Probability of infection across treatments, bars denote 95% confidence intervals, (b) relationship between the predicted probability of infection and simulated hatching dates obtained from the model, (c) relationship between the predicted probability of infections and simulated brood sizes obtained from the model.

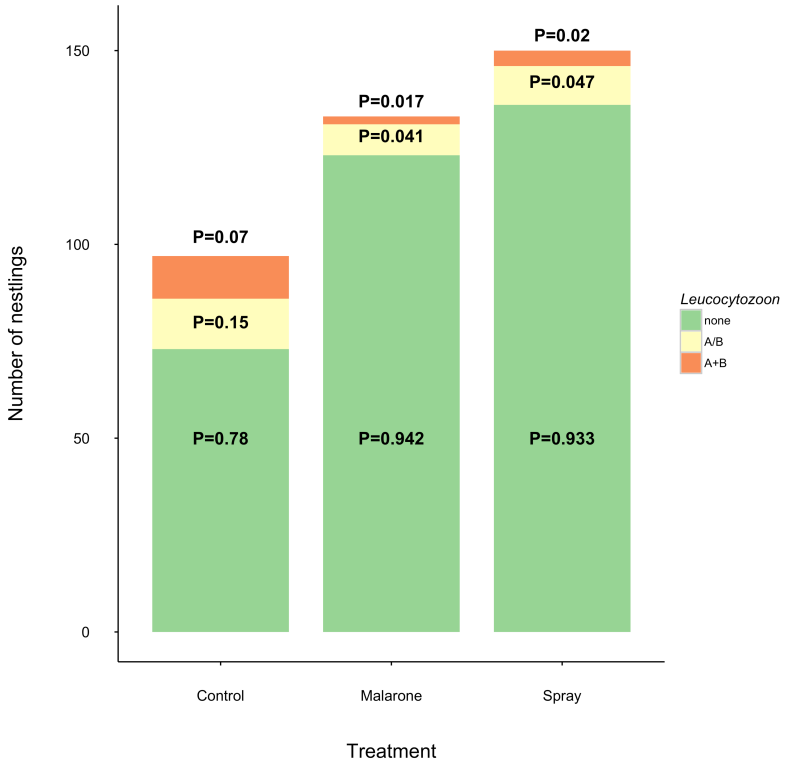


Figure 3. Number of nestlings infected with none, one or two *Leucocytozoon* haplotypes across treatments. Predicted probabilities obtained from the ordinal mixed model are shown above bars.

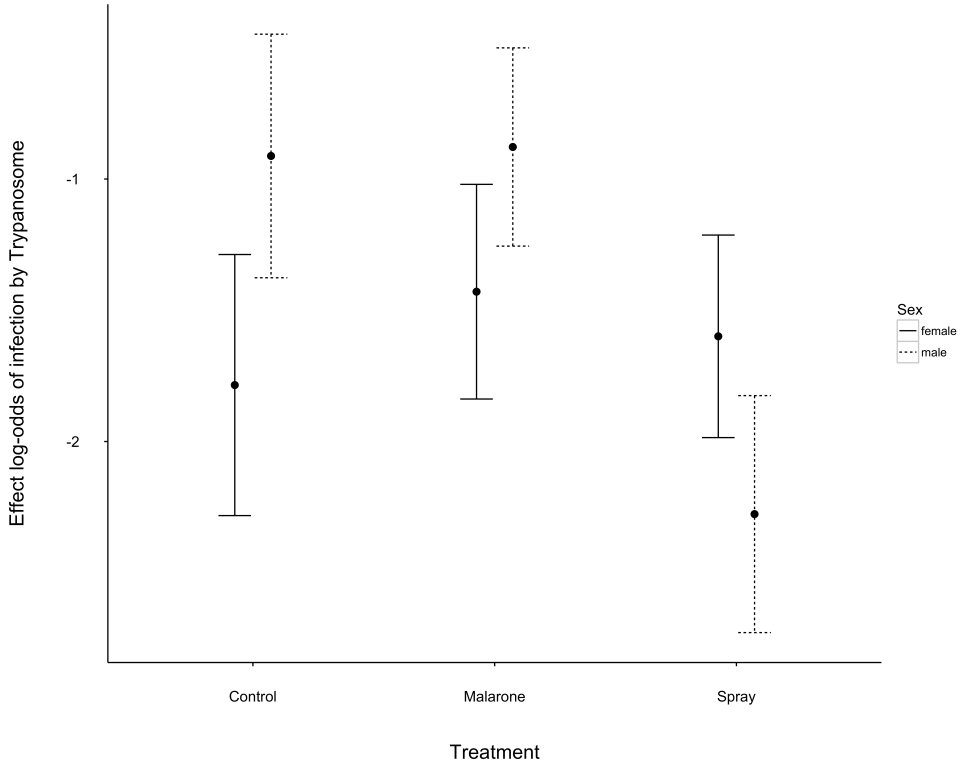


Figure 4. Log-odds ratio of infection by *Trypanosoma* spp. derived from Bayesian estimates across treatment groups and sexes. Bars denote 95% confidence intervals.

Table 2. Generalised linear mixed models for endoparasites infecting blue tits' nestlings. Presented are the mean and credible 95% confidence intervals extracted from Bayesian mixed models. Significant p-values are highlighted in bold. Codes: AIC (Akaike Information Criterion), df= degrees of freedom.

Parasite	Predictor variable	Bayesian Estimate	Bayesian p-value	AIC (df)	R^2 (%)	Prevalence (%)	Sample size
Leucocytozoon A	Treatment Control vs.			190.49(7)	25.3	9.45	380(71)
	Malarone	-1.49(-2.78, -0.08)	**0.0202**				
	Insecticide	-1.30(-2.61, -0.04)	**0.0425**				
	Hatching date	-0.11(-0.22, -0.01)	**0.0142**				
	Brood size	0.33(0.06, 0.62)	**0.0142**				
	***Leucocytozoon* B**	3.49(2.31, 4.75)	**<0.001**				
Leucocytozoon B	Treatment Control vs.			152.45(6)	24.0	8.02	380(71)
	Malarone	-1.15(-2.90, 0.72)	0.21				
	Insecticide	-0.94(-2.92, 0.68)	0.26				
	Relative growth	0.96(-0.15, 2.14)	0.11				
	***Leucocytozoon* A**	3.86(2.72, 5.21)	**<0.001**				
Leucocytozoon A and B	Treatment Control vs.			334.26(9)	-	-	380(71)
	Malarone	-1.20(-1.96, -0.32)	**0.00405**				
	Insecticide	-1.12(-1.94, -0.37)	**0.00607**				
	Sex	-0.37(-0.92, 0.14)	0.17				
	Hatching date	-0.07(-0.13, -0.01)	**0.01417**				
	Relative growth	0.29(-0.20, 0.79)	0.27				
	Brood size	0.15(-0.01, 0.31)	0.0749.				
Trypanosoma spp.	Treatment Control vs.			396.86(9)	4.04	23.35	380(71)
	Malarone	0.53(-1.07, 2.19)	0.53				
	Insecticide	0.34(-1.12, 2.17)	0.70				
	Sex	1.13(-0.17, 2.58)	0.10				
	Treatment (MAL)*Sex(M)	-0.41(-2.20, 1.23)	0.63				
	Treatment (SPR)*Sex(M)	-1.96(-3.72, -0.27)	**0.02834**				
	Hatching date	0.06(-0.02, 0.13)	0.10				
	Wing growth	0.12(0.002, 0.24)	0.05061.				

Effect of the treatment on feather colouration

Breast colour was not related to the treatment or other breeding parameters in nestlings (linear mixed effect model, all P-value>0.05). Luminance in the yellow breast, however, was significantly higher in male nestlings ($F_{1, 332.64}$=3.99, P-value=0.046, effect size ES=0.25), but this was independent of treatment or other breeding parameters (linear mixed effect model, all P-value>0.05). Tail saturation was different across treatments ($F_{2, 98.74}$=3.34, P-value=0.039, effect size ES=0.17): nestlings from the insecticide-sprayed

group had less saturated green tail feathers (Student's t=2.54, P-value=0.012, Fig. 5a). Heavier nestlings ($F_{1, 315.97}$=6.02, P-value=0.015, effect size ES=0.20) and nestlings reared in nests that hatched later in the season ($F_{1, 80.57}$=8.69, P-value=0.0042, effect size ES=0.24) were also significantly less saturated in their tail feathers. Tail luminance was not related to the treatment, sex, brood size or the interaction between treatment and sex (linear mixed effect model, all P-value>0.05), but it was positively related to later hatching date ($F_{1, 74.27}$=17.18, P-value<0.0001, effect size ES=0.30). There was a trend for increased tail brightness with more relative growth ($F_{1, 311.23}$=3.22, P-value=0.074, effect size ES=0.13) and nestling body mass on day 15 ($F_{1, 304.89}$=0.089, P-value=0.079, effect size ES=0.13).

In winter, 15 nestlings were recaptured as yearlings, and colour JND scores were used to compare the change in feather colour between developing nestlings and first-year individuals (yellow breast and blue-green base of the tail). We found no effect of the treatment administered in the nest on colour change (Welch two-sample t-test, all P-value>0.05, N=15, Table A1). Additionally, colour variables in the white cheek and the blue crown were related to the treatment administered during the nestling stage. First-years from the insecticide sprayed group developed brighter blue crowns (Welch t-test= -2.98, df= 6, P-value= 0.02521, Effect size ES=1.81, N=15, Fig. 5b).

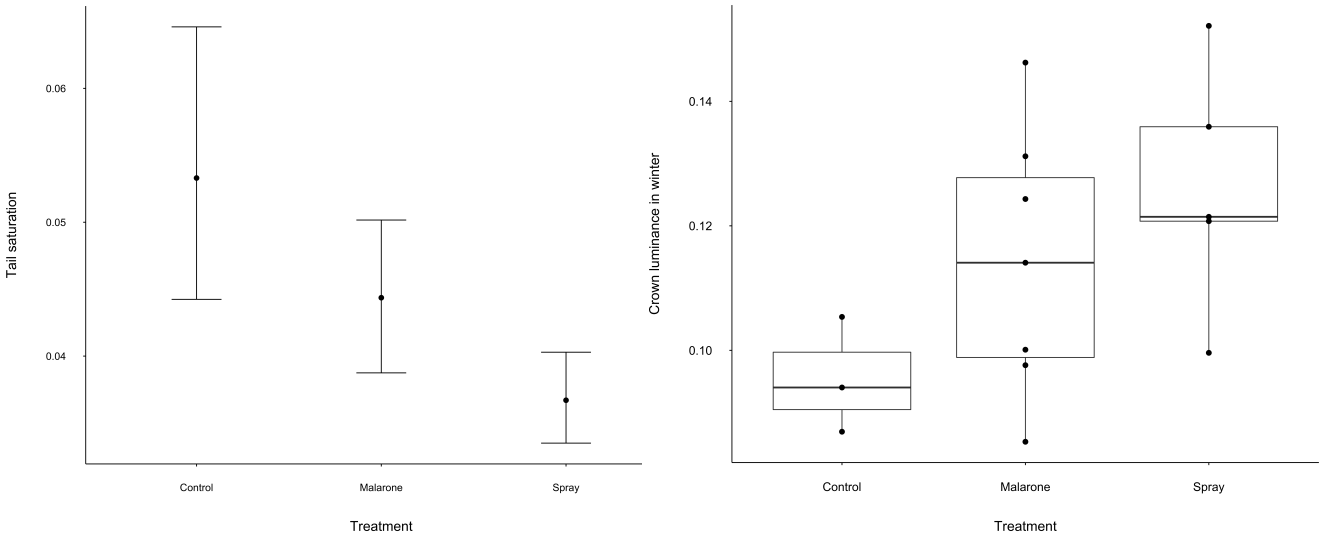


Figure 5. a) Saturation in the green tail feathers of nestling blue tits across treatment groups. Bars denote 95% confidence intervals; b) Crown brightness across treatment groups in first-year individuals captured in winter. Shown are medians (solid horizontal line), non-outlier range (whiskers), and interquartile range 25–75% points in the cumulative distribution (rectangles).

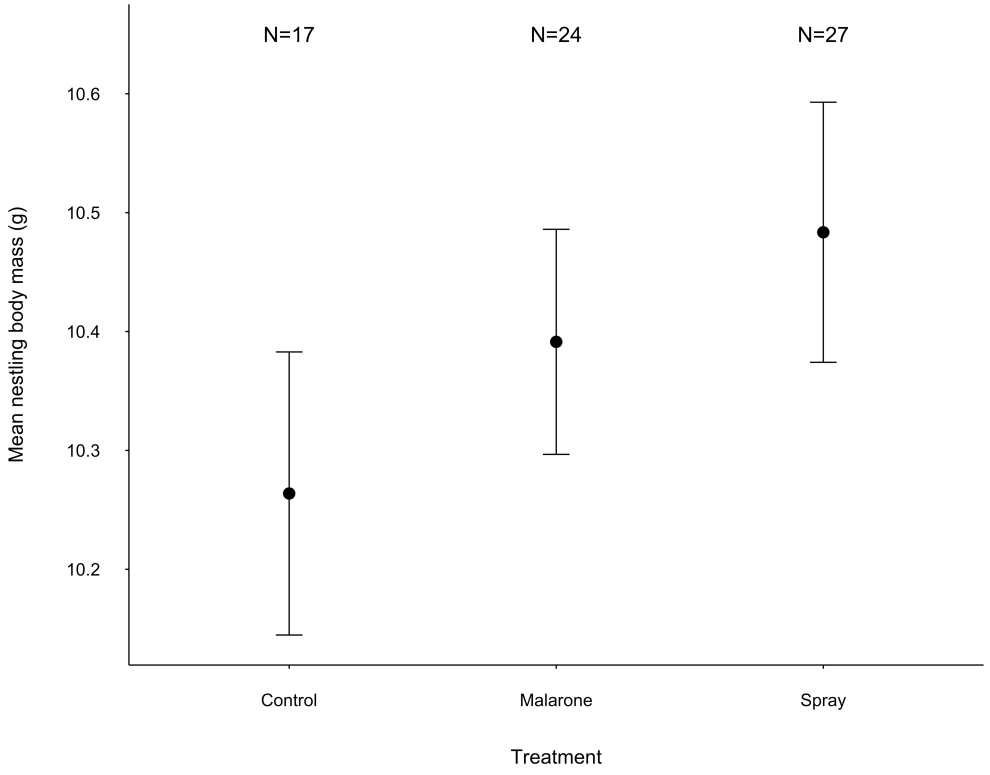


Figure 4. Mean blue tit body mass (g) before fledgling across treatment groups. Bars denote 95% confidence intervals.

Infections by haematozoans and extra-pair paternity

The probability of being infected by *Leucocytozoon* A (Exact test, Z=1.19, P-value=0.13, N=518) or *Trypanosoma* spp. (Exact test, Z=-0.21, P-value=0.99, N=518) was independent of extra-pair paternity. The odds of being infected by *Leucocytozoon* B were higher for extra-pair nestlings (Odds ratio=0.17 vs. 0.074, Exact test, Z=2.47, P-value=0.015, N=518 total number of nestlings, N=96 extra-pair nestlings). Infections were independent of nestling sex. Sex was independent of paternity (Exact test, Z=0.49, P-value=0.36, N=518, N=96 extra-pair nestlings).

Effect of the treatment on breeding parameters

When using nestling ID as unit of analysis we found no differences across treatments in body mass at fledgling (Linear mixed effect model, $F_{2, 83.84}$=1.20, P-value=0.3, N=380). Variation in fledgling body mass was mostly explained by sex differences: male nestlings were significantly heavier than female nestlings ($F_{1,335.09}$=14.29, P-value=0.0002, Effect size ES=0.57, N=380). However, when mean nestling body mass was tested across nests, the most parsimonious model included the treatment and the interaction between the abundance of biting midges and the presence/absence of blackflies (AIC=-90.62). Mean fledgling body mass tended to be higher in the insecticide-sprayed nests (Student's t=1.96, p-value=0.05463, Fig. 6). In nests with higher biting midges' abundance and presence of blackflies, mean nestling body mass was significantly lower (Student's t=-2.66, p-value=0.01012). Fledgling success was not different across treatments (GLM binomial, χ^2_2=95.46, P-value=0.22, N=74).

DISCUSSION

As a result of our experimental manipulation, blue tit nestlings from the insecticide-sprayed group were less likely to be infected by blood parasites and were reared in nests that harboured less ectoparasites and than those from the control group.

At fledgling, these nestlings were less saturated in their blue-green tail feathers and tended to have increased body mass.

The spraying treatment was effective in reducing the entire ectoparasite community in blue tits' nests, in accordance with previous experimental studies in the present population using the same insecticide (biting midges in Martínez-de la Puente et al. 2009b; nest-dwelling parasites in Tomás et al. 2007; Martínez-de la Puente et al. 2011). Additionally, in the 2013-breeding season later broods were infected with more parasites because the warmer climates during the end of the breeding are favourable for flying insects in temperate regions (Tomás et al. 2008; Arriero et al. 2008; Martínez-de la Puente et al. 2009b; Cantarero et al. 2013). We also found that nests with larger broods were more infected by blackflies, also in agreement with another study in the present population (Rivero-De Aguilar et al. 2016). The higher release of substances that are attractive to flying parasites in crowded nests may be an explanation for the presence of higher parasite loads (Martínez–de la Puente et al. 2009).

The treatment was also effective in reducing the endoparasite community in the medicated and insecticide-sprayed groups of nests. The odds of remaining uninfected by any *Leucocytozoon* haplotype were higher if nestlings were reared in the medicated or the insecticide-sprayed group (see Fig. 3; ordinal model). Strikingly, the treatment did not appear to be effective against *Leucocytozoon* haplotype B when haplotypes A and B were tested separately. This could be due to drug resistance, which has been reported when treating human malaria the virulent *Plasmodium falciparum* (Pukrittayakamee et al. 2000). In blue tits, *Leucocytozoon* B may become more virulent when the host is simultaneously infected by haplotype A. High intensities of infection would make it difficult to eliminate both haplotypes from blood (Marzal et al. 2008). Another possibility could explain the differential effectiveness of the treatment against infections by *Leucocytozoon* A and B. Nestling blue tits may carry MHC genes that confer

susceptibility/resistance to infections by *Leucocytozoon* B, which has been suggested recently in our population for this species (Rivero-de Aguilar et al. 2016). A resistant MHC allele would maintain low infection intensities but avoid the lethal effects of the parasite, whereas a susceptible MHC allele may confer resistance to an undetected parasite (Westerdahl et al. 2012). In fact, MHC genes can alter the competitive interactions between malarial parasites within the host (Loiseau et al. 2008). Our results concerning paternity are in line with this hypothesis, because extra-pair nestlings were more likely to harbour infections by *Leucocytozoon* B, and therefore hint at genetic effects in parasite resistance/susceptibility (Merino et al. 1996; Saino et al. 2002).

Infections by the haematozoan parasite *Trypanosoma* spp., were not affected by the anti-malarial medication, which is not unexpected because Malarone™ does not specifically target this blood parasite. However, they were reduced by the insecticide treatment, albeit only in male nestlings. Sex-specific differences in body condition may explain the different patterns observed in susceptibility to infection. Male nestling blue tits were heavier at fledgling and more saturated in their yellow plumage, which indicate that they may develop better immune responses. Still, our results suggest that under favourable conditions for vectors, these may feed on individuals in better body condition, in agreement with other studies in birds and mammals (Valera et al. 2004; Witsenburg et al. 2015). On the contrary, under negative conditions (nests sprayed with insecticide), vectors may feed on individuals in poor condition (females in this study), probably due to their weakened immune system responses (Merino et al. 1996), in agreement with the 'tasty-chick hypothesis (Christe et al. 1998). Vector feeding preferences may also vary according to host availability (Santiago-Alarcon et al. 2012), or in some cases, they may even be driven by parasitic infections in the host (Cornet et al. 2013). Whether this is the case with trypanosomes remains unknown.

Nonetheless, reducing the ectoparasites' and endoparasites' community from blue tit nests had a significant effect on the structural ornament, the green tail. Nestlings from the insecticide-spray group of nests grew less saturated green tail feathers. One possibility is that uninfected nestlings were exposed to degradation in their green-blue tail feathers due to higher mobility inside the nest. Indeed, increased begging and overall activity has been suggested to increase feather wear in nestling blue tits (Jacot and Kempenaers 2006), which may ultimately reduce tail saturation. Even small changes in the arrangement pattern of the feather's microstructure can explain variation in plumage colour (Shawkey et al. 2003), for instance, in the structural fraction of the green-blue tail in nestling blue tits.

The implications of reduced saturation in the blue-green tail feathers during the nestling stage are unknown, but it is unlikely that it serves a parent-offspring communication purpose because the base of the tail is usually not exposed in the nest. Another possibility is that they may convey information about future reproductive success, since tail feathers developed at the nestling stage are maintained during the individuals' first reproductive event. Peters et al. (2007) reported sexual dimorphism in UV reflectance in nestling blue tits, but the authors were based on differences in reflectance in the ultraviolet range of the spectrum. Here, we used an objective and more biologically informative avian vision model but we could not find evidences for sexual dimorphism in the structural colouration of the blue-green tail in nestling blue tits. Still, tail saturation could covary with another ornament that is sexually selected, but this requires further confirmation from behavioural experiments. Confirmation for this hypothesis in our study is lacking: if less saturation is preferred by prospective mates, nestlings from the sprayed groups of nests should have changed their tail colouration significantly less between seasons than nestlings from the control group, but this was not the case (Table A1).

Notwithstanding, colour expression in the sexually selected blue tit crown was affected by the treatment. In our study population, first-years from the insecticide group grew brighter blue crowns, which confirms the idea that prospective mates may prefer mating with individuals reared in parasite-free nests. To our knowledge, this is the first experimental study showing that reduced parasite loads in early-life positively affect feather colouration in a structural ornament that is moulted shortly after leaving the nest.

Contrary to our expectations, the treatment had no effect on the nestlings' yellow plumage colouration. Three hypotheses could explain the lack of effect of both the medication and insecticide on the carotenoid-based ornament. First, the sub-curative dose e might not be sufficient to increase the nestlings' health status because parasitic infections may not be completely eliminated (Foronda et al. 2007). Second, other parasites that remained undetected might infect nestling blue tits. Malarone™ effectively removed the malarial parasite *Plasmodium relictum* in adult blue tits from other European populations (Knowles et al. 2010), but its effect on other malaria-like parasites are unknown. Unfortunately, infections by other genera (i.e. *Haemoproteus* spp.) need longer prepatent periods (15 days), as opposed to parasites like *Leucocytozoon* and *Trypanosoma* spp., which have shorter prepatent periods (5-6 days, Merino & Potti, 1995). Thus, undetected infections by *Haemoproteus* could have hindered the positive effects of the treatment on the yellow breast colouration. In fact, negative effects on yellow plumage have been reported when adult blue tits were infected by several parasite species in this study population (del Cerro et al. 2010). The third hypothesis explaining the lack of effect of the treatment on carotenoid-based colouration could be related to undetected mild side effects from the treatment. In nestling blue tits, however, no negative effects on body mass or fledgling success were observed after the administration of Malarone™. Additionally, the insecticide used in our study has safely been used in this study population before (Tomás et al. 2007; Martínez-de la Puente et al. 2011), so side effects are unlikely. In fact, in the spring of 2013, nestlings reared in sprayed nests tended to be heavier. Still, other

effects (i.e. on carotenoid deposition in yellow breast feathers) related to the use of these chemicals during bird development remain unknown.

In conclusion, our results support the hypothesis that parasites impose physiological costs for cavity-nesting birds during development, and that these costs may affect feather colouration in an apparently unexpected direction. Nestling blue tits tended to have increased body mass at fledgling when they were reared in nests that had less ectoparasites, lower likelihood of becoming infected by *Leucocytozoon* in both sexes, and lower likelihood of becoming infected by trypanosomes in males. Those nestlings grew less saturated green-blue tail feathers, but the implications of this trait in future fitness in blue tits is unknown. Additionally, fewer infections by blood parasites and ectoparasites in early life result in increased brightness in another sexual ornament that is relevant for mating. Potential partners in the following reproductive season may prefer to mate with individuals showing brighter blue crowns and less saturated green tails. In the blue tit, the information content from two structural ornaments may indicate development in a favourable environment in early life to conspecifics. Future studies will benefit from taking into account the developmental conditions experienced at the nest, as these may explain mating patterns and reproductive success, at least in the following breeding season.

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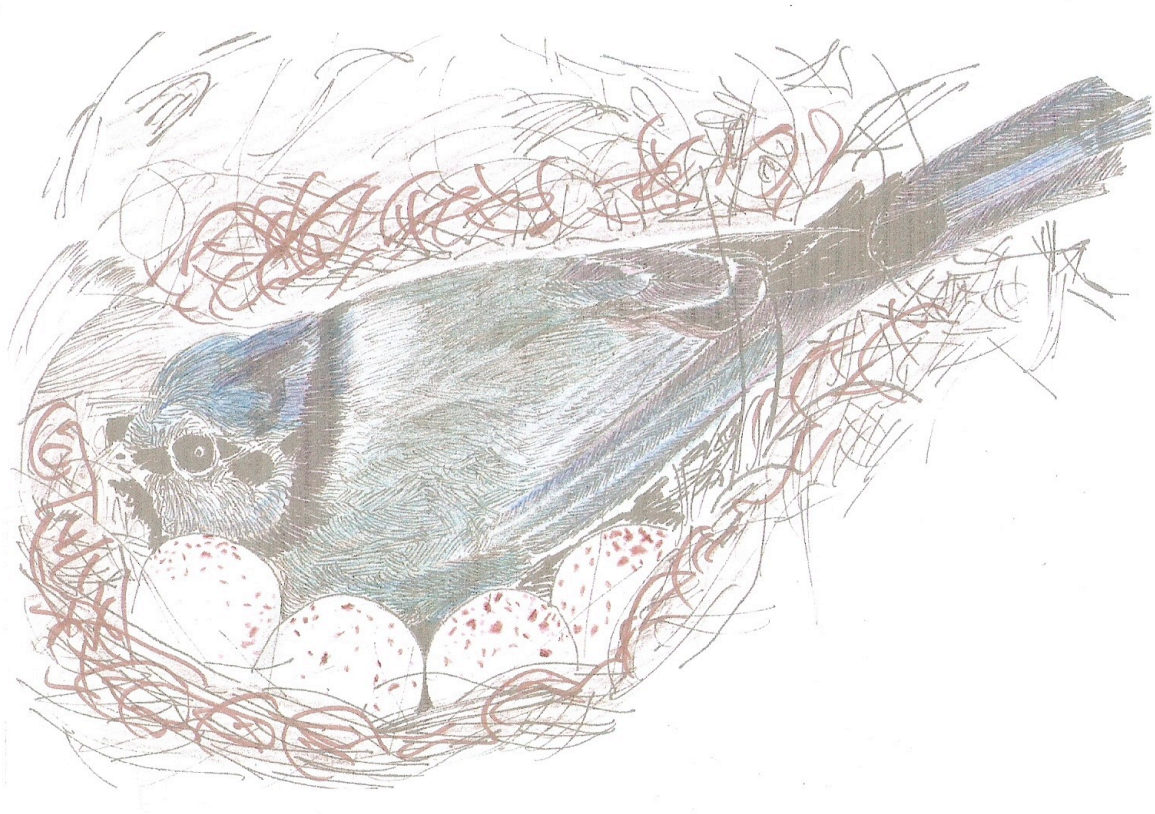
PART II

MATING AND PATERNITY

Chapter 3



'In nature there are neither rewards nor punishments; there are consequences.'

—Robert Green Ingersoll

This chapter reproduces entirely the manuscript:

E.P. Badás, J. Martínez, J. Rivero-de Aguilar, M. Stevens, M. van der Velde, J. Komdeur and S. Merino (2017) Eggshell pigmentation in the blue tit: male quality matters. *Behavioral Ecology and Sociobiology* **71**:57.

Eggshell pigmentation in the blue tit: male quality matters

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Abstract Many passerines lay protoporphyrin-pigmented eggs, and the degree of spotting seems to be related to female condition and environmental characteristics. However, most studies have ignored the relationship between the male's quality and eggshell pigmentation. Because ornaments can act as honest indicators of individual quality, spottiness could be related to the parents' feather colouration. Using models of bird vision, we investigated whether male and female ornamentation explained variation in spotting coverage in a free-living population of blue tits (*Cyanistes caeruleus*). We also explored the associations between other important individual characteristics (i.e. the pair's infection status) and spotting coverage. Females that laid more pigmented eggs suffered from higher parasitaemia by the blood parasite *Leucocytozoon*, had smaller clutches, more saturated yellow breasts feathers and reduced body mass. Male plumage colour and infection status explained a higher percentage of the variation in eggshell pigmentation than female characteristics. Males with more saturated white cheeks, less saturated yellow breasts, more intensely infected by the parasite *Haemoproteus* and less by *Plasmodium*, attended nests with more spotted eggs. Additionally, these males were younger and more likely to father extra-pair offspring. These results, although observational, suggest that male attractiveness, male age, extra-pair paternity, and parasitic infections could be important determinants in eggshell pigmentation. Males in poorer condition might have provided less food to laying females, which in turn laid more pigmented eggs and were also in poor condition. Alternatively, increased eggshell pigmentation could result from female differential allocation or breeding in low quality territories.

Keywords age, avian malaria, feather colouration, paternity, protoporphyrin

INTRODUCTION

Females of many passerine species lay eggs with reddish-brown spots that are pigmented by natural porphyrins (McGraw 2006a). These pigments could play a major role in ensuring the successful development of the avian embryo (Maurer et al. 2011; Lahti and Ardia 2016). In passerines, variation in maculation has a significant genetic basis (Gosler et al. 2000), but the pattern and quantity of spots is also affected by female condition (Moreno and Osorno 2003; Martínez-de la Puente et al. 2007; Giordano et al. 2015), laying order (De Coster et al. 2013), and calcium availability (Gosler et al. 2005; García-Navas et al. 2011). Even within a single species, there can be great variation in eggshell spottiness between female clutches (Gosler et al. 2000). Differences in pigment deposition with respect to the male's characteristics, however, remain unclear.

In species with male courtship feeding, female condition could be influenced by their mates' ability to provide food (Otter et al. 2007; Martínez-Padilla et al. 2010). In fact, males may invest differently in courtship feeding depending on the female's characteristics, which in turn could affect female condition and egg laying. The relationship between spottiness and male quality in the wild has been explored, but yielded contradictory results. Studies in passerines have shown that females laid more pigmented eggs when mated to lower quality males (Martínez-de la Puente et al. 2007; Hargitai et al. 2008), whereas a study on Eurasian kestrel (*Falco tinnunculus*) found the opposite: more ornamented females laid more spotted eggs when mated to males in better condition (Martínez-Padilla et al. 2010). In blue tits (*Cyanistes caeruleus*), pigment distribution on the eggshell has been shown to be positively associated with male provisioning rates and male body mass (Sanz and García-Navas 2009). However, additional studies exploring whether multiple male characteristics relate to eggshell pigmentation are needed.

The relationship between female quality and eggshell pigmentation has been extensively studied, but it is far from clear. The female's nutritional condition is particularly important during egg formation and egg laying, and it can affect egg characteristics. Eggshell maculation is likely to be related to female condition due to the pro-oxidant nature of protoporphyrin pigments (Afonso et al. 1999). For example, female Japanese quail (*Coturnix coturnix japonica*) in lower body condition after food deprivation allocated more protoporphyrin pigments to the eggshell (Duval et al. 2013b). However, the authors of this study found no effect of the treatment or the female's condition on the spots' reflectance spectra (Duval et al. 2013b). Several studies have found that associations between eggshell pigmentation and female condition may relate to calcium availability and eggshell thickness (see Cherry and Gosler 2010 for a review). Still, different components of eggshell pigmentation may provide different information. In great tits (*Parus major*), females with higher blood antioxidant capacity laid eggs with more evenly distributed spots (pigment spread) and higher yolk antioxidant capacity (Giordano et al. 2015). Other studies, also in great tits, found no relationship between pigment spread and female condition, and instead reported that pigmentation darkness was negatively affected by parasite load (De Coster et al. 2012), and positively related to female quality (De Coster et al. 2013). In contrast, in the same species, Hargitai et al. (2016) found a negative association between pigment darkness and female quality. In blue tits, pigment spread has been positively related to egg characteristics that may indicate higher female quality (Sanz and García-Navas 2009) and maternal investment in yolk antibodies (Holveck et al. 2012); while pigment darkness was positively related to female body size (Sanz and García-Navas 2009). Thus, the relationship between egg pigmentation and individual quality requires further investigation in both members of the pair.

Ornaments can provide information about an individual's condition because the production and maintenance of ornaments imposes both physiological and survival costs (Møller and Szép 2002; McGraw 2006b). Additionally, the Hamilton and Zuk (1982)

parasite-mediated sexual selection hypothesis proposed that individuals bearing the most exaggerated sexual trait signal higher resistance to parasites. Indeed, carotenoid colouration can be used as a proxy for individual quality, as it depends on access to carotenoids through diet and nutritional condition (McGraw et al. 2005), and it can be related to parasitic infections (Hill 2006b). Structural colours of feathers can also be condition-dependent (for a review, Hill 2006a). The relationship, if any, between achromatic colour patches and condition is, however, less studied (Prum 2006). There is recent evidence for condition-dependence of white colouration (Ferns and Hinsley 2004; Hanssen et al. 2006; Moreno et al. 2011), but it is inconclusive (Griggio et al. 2009). In order to study the functional basis of avian colouration it is essential to measure quality in terms of avian vision by using models that take into account the receiver's visual perception (Endler and Mielke 2005; Stevens 2011). This approach is the most biologically informed with regards to how avian colour vision likely works and is based on a widely adopted and implemented tetrahedral colour space approach that is available in the literature at the moment (Stoddard and Prum 2008). An increasing number of bird colouration studies use colour vision metrics calculated from colour spaces in order to reveal how the colour properties of a given receiver may vary with other traits (i.e. eggshell pigmentation) (Walker et al. 2013; Dakin et al. 2016; Trigo and Mota 2016).

Promiscuity has also been related to individual quality because males can enhance their reproductive success by mating with several females (Trivers 1972). In recent decades, attention has focused on how females actively seek extra-pair matings (Charmantier et al. 2004), but male characteristics in relation to extra-pair paternity have also been intensively studied (Delhey et al. 2007; Forstmeier et al. 2014). However, it is still unclear whether eggshell pigmentation relates to paternity. If eggshell pigmentation is related to some aspect of female condition, it could also be related to males seeking extra-pair fertilizations.

In this study, we explored the relationship between eggshell pigmentation and individual quality of breeding blue tits (*Cyanistes caeruleus*). As explained above, eggshell pigmentation variables such as pigment spread and pigment darkness (assessed as Gosler et al. 2000) are not equally related to female characteristics. Thus, we used spotting coverage as a measure of eggshell pigmentation. Recently, it has been reported that the area covered by spots is highly correlated with protoporphyrin concentration in the eggshell (Wegmann et al. 2015; Hargitai et al. 2016). Although this was found in great tits, pigment deposition and eggshell patterning are very similar in blue tits (Flanagan and Morris 1975). In addition, spotting coverage has been shown to correlate well with female quality in our study population (Martínez-de la Puente et al. 2007), and it positively correlated with pigment spread in a blue tit population located nearby (Sanz and García-Navas 2009). We used ornamentation, parasitic infections and extra-pair paternity as indicators of individual quality. Following the increasing number of bird colouration studies, we will use colour vision metrics to explore the relationship between eggshell pigmentation and plumage colouration. In the blue tit, ornamentation in the ultraviolet (UV) blue crown and the yellow breast feathers has already been used as a proxy for individual quality (Doutrelant et al. 2008). Blue tits also feature sexually dichromatic UV colouration in the white cheek, albeit its signalling function remains unclear (Griggio et al. 2009). Furthermore, the population under study is infected by several avian malaria-like parasites with harmful effects on reproductive success (Merino et al. 2000; Martínez-de la Puente et al. 2010) and carotenoid colouration (del Cerro et al. 2010). Structural colouration of feathers is an indicator of the history of parasitism in other bird species (Hill 2006a), but to date, a direct relationship between blood parasites and eggshell spottiness has not been reported (although see Hargitai et al. 2016). In the blue tit, structural crown colouration has been related to individual quality and extra-pair paternity before (Delhey et al. 2007). Because crown colouration could not be measured in this study (see Methods section), we explored the relationship between paternity and

spotting coverage instead. Blue tits are socially monogamous, but some pairs frequently engage in extra-pair copulations (Kempenaers and Schlicht 2010). The rate of extra-pair paternity in the present population is unknown, but in another blue tit population located nearby nearly half of the nests contained extra-pair nestlings (García-Navas et al. 2014). Finally, we also included other reproductive factors (body condition, clutch size and hatching date) and age as predictors of spotting coverage, as these may explain variation in eggshell pigmentation (López-Rull et al. 2007; Cherry and Gosler 2010).

The main specific aims of this study were: 1) to examine whether variation in eggshell pigmentation is related to secondary sexual characters (i.e. plumage colouration) and infection status in order to identify which characteristics from the breeding pair explain most of the variation in spottiness; and 2) to test whether extra-pair paternity and cuckoldry correlate with variation in pigmentation. Secondary aims were to explore whether the female and her partner's age predict eggshell pigmentation, and to test breeding phenology and reproductive trait effects on spottiness. If poorer quality females lay more spotted eggs, we expect that their male mates engage in more extra pair copulations and suffer from less cuckoldry. Poorer quality females would bear the costs of parasitic infections by showing duller plumage ornaments, and pairing with lower quality males. Alternatively, if females in better condition lay more pigmented eggs, males may invest more in their social pair, and consequently, the relationship between extra-pair paternity and eggshell pigmentation should be the opposite. Higher female and male quality may be seen in less intense infections by parasites and more exaggerated colour traits. To our knowledge, we combined, for the first time, ornamental cues and condition variables in order to elucidate which female or male characteristics explained a higher proportion of the variation in eggshell pigmentation.

METHODS

Study site and sampling

The study was conducted during the spring of 2012 on a population of blue tits breeding in a deciduous forest of Pyrenean oak (*Quercus pyrenaica*) in the vicinity of Valsaín (Segovia), central Spain (40°53'N, 4°01'W, 1200 m.a.s.l.), where 300 wooden nestboxes are available. Blue tits occupy an average of 25% nestboxes per year (Merino et al. 1997).

Nests were visited daily during laying, and the photographs were taken at the onset of incubation in order to avoid ectoparasite bite marks on the eggshell, which can increase during incubation (Avilés et al. 2009). The day after the last egg was laid, we carefully extracted all the eggs of the entire clutch from the nest and placed them in a soft foam rubber holder with grey background colour. Using an umbrella to shade the whole clutch, each egg was then photographed with a Canon IXUS 130 camera. Because we were not interested in the size or colour properties of the spots, but instead in the proportion of spottiness covering the egg surface, the distance between the camera and the egg and the varying light conditions were not limiting factors when taking photographs.

Figure 1. Field set-up for clutch photographs. a) Eggs were carefully removed from the nest and placed on a soft mat. b) Egg close-up showing protoporphyrin pigmentation.

When nestlings were 3-days old (hatching date = day 0), adults were captured at the nestbox. On day 15, nestlings were ringed and bled for paternity analyses. Adult birds were ringed, weighed to the nearest 0.1 g and aged according to plumage characteristics as yearlings or adults (i.e.: two or more years old, see Svensson 1992). Wing (±0.5 mm; method III following Svensson, 1992) and tarsus (±0.1 mm) length were also recorded. Mass was corrected by regression for body size (tarsus length) and time of day (Senar 2002). Female mass was not measured during the incubation or laying period so as to prevent nest desertion, but we assume that body condition of laying females is closely related to their condition when nestlings are 3 days old. No female desertion was found after manipulation. A blood sample was obtained via the brachial vein. One drop of blood was stored on an FTA card (Whatman, UK) for molecular analyses (parasitological analyses and paternity, see below). We also measured feather colour reflectance on two different patches: yellow breast in males and females, and white cheek in males. Although blue tits are sexually dimorphic on the white cheek (Griggio et al. 2009), we measured colour only in males in order to reduce handling time of the female and thus avoid nest desertion. Colour spectra were collected using a spectrophotometer (Ocean Optics Inc., Dunedin, FL, USA) connected to an Ocean Optics fibre-optic reflection probe. The probe was made up of seven optical fibres that were illuminated by a Pulsed Xenon Light Source (Jaz-PX lamp) and it was inserted in a miniature black chamber that acted as holder and excluded ambient light. The equipment was calibrated with a flat white standard (Ocean Optics) prior to each bird measured. Reflectance data from 300 to 700 nm were undertaken at 90º incidence and 3 mm from the feather surface over an illuminated circular area approximately 1 mm in diameter. Each spectrum was an average of three scans and was calculated relative to the reflectance produced by the white standard and a dark current. The probe was lifted between repeated measurements within a body region. The blue feathers of the blue tit crown are also sexually dimorphic in the UV, and their brightness has proved to signal male quality (Sheldon et al. 1999). However, in this study,

colour data for the blue crown were discarded due to technical difficulties (feather bristling in this patch could not be avoided with the holder provided by the manufacturer, see above). Additionally, some individuals had lost their crown feathers due to fights, thus making it difficult to position the holder.

Eggshell pigmentation scoring

Blue tits lay white eggs maculated with protoporphyrin-based red-brown spots (Martínez-de la Puente et al. 2007). Eggshell pigmentation was scored as spotting coverage using Adobe Photoshop CS (v. 5.1). In each egg we measured the total area occupied by spots in pixels with respect to the total area of white in pixels. This method is highly repeatable in our study population (Martínez-de la Puente et al. 2007). Because the intra-clutch coefficient of variation was low ($CV_{intra\text{-}clutch}$=4.1 vs. $CV_{inter\text{-}clutch}$=9.5, $F_{40,237}$ = 5.37, N = 41, P<0.0001), egg pigmentation values were averaged per nest and clutch size was included as an explanatory variable in the subsequent analyses. One observer scored 390 eggs from 41 clutches (EPB), those where we were able to collect colour and parasite data from both members of the social pair (see below). To minimize observer bias, blinded methods were used when pigmentation data were collected.

Parasite quantification

For all samples, DNA was extracted from blood using a standard ammonium-acetate protocol and stored at -20ºC. This DNA solution was then purified using silica filters to obtain a higher quality DNA (NZYGel pure, NZYtech, Lda. -Genes and Enzymes). DNA samples were quantified by spectrophotometry and adjusted to the same concentration (10ng/uL). We detected and quantified the following parasites using quantitative PCR (qPCR) with SYBR green (SYBR Selected Master Mix, Applied Biosystems) to amplify a fragment of the cytochrome B or 18S rRNA genes using a pair of species-specific primers for each parasite: *Haemoproteus majoris* haplotype cyan2,

Plasmodium spp. haplotype cyan1, *Lankesterella valsainiensis*, *Leucocytozoon majoris* haplotypes leuA, leuA1 and leuB. The variable *Leucocytozoon* A includes haplotypes A and A1 (see Badás et al. 2015 for more information on the primers used).

Molecular parentage analyses

Parents and nestlings were genotyped for eight microsatellite loci; information on microsatellites, primers and polymerase chain reaction (PCR) conditions are detailed in Table 2 in the **General Methods Chapter**. PCRs were carried out in 10 µl volume using a QIAGEN Multiplex PCR Kit (Qiagen, Valencia, CA) and 20-50 ng of template DNA. Fluorescently labelled PCR products were separated on an AB3730 DNA analyser. Subsequently allele lengths were determined using Genemapper 4.0 software. For each nestling the microsatellite genotypes were compared with its social father, and the offspring was assigned as extra-pair if there were at least two mismatches between the genotype of the social father and offspring. Extra-pair paternity (EPP) was assigned when one of the sampled males matched all of the offspring's paternal alleles. Paternity was assigned for 81% of all identified extra-pair fledglings (genotyped nestlings N=607, extra-pair nestlings N=84, assigned EP nestlings=68; 79 nests) in the population using Cervus 3.0 (Kalinowski et al. 2007). Maternity of the social female was confirmed by the microsatellite data for all nestlings. The mean exclusion probability of the eight markers was calculated to be 0.99986 for the first (female) parent and 0.99999 for the second (male) parent (given the genotype of the first parent).

Models of bird vision

Colour vision in birds likely stems from four single cone types (Cuthill 2006), while luminance-based tasks are widely thought to stem from the double cones (Jones and Osorio 2004). To model the UV-sensitive (UVS) blue tit visual system, we used their known photoreceptor spectral sensitivities (Hart et al. 2000) and extracted hue,

saturation, and luminance variables for each colour patch (Endler and Mielke 2005; Stevens et al. 2009) (see Appendix, Figs. A1 and A2). Based on a standard daylight 'd65' irradiance level (Vorobyev et al. 1998), we processed the reflectance spectra for each patch in order to calculate the relative quantum (photon) catch values for the four single cones, used in colour vision, and the double cones, used in luminance vision (Endler and Mielke 2005; Stevens et al. 2009). Our measure of luminance, the perceived lightness of a patch (brightness), was simply the double cone photon catch values. To calculate saturation, the amount of colour compared with white light, we plotted the standardized single cone catch data for each individual in avian tetrahedral colour space (Stevens et al. 2009) and calculated the distance from the centre of the colour space (following Endler and Mielke 2005). To calculate hue or colour type for the yellow patch, we derived colour channels based on using ratios from the photon catch outputs. This approach is broadly inspired by the way that opponent colour channels work in vision in encoding antagonistic colour types (Osorio et al. 1999) and is based on recent work following the same methods (Komdeur et al. 2005; Spottiswoode and Stevens 2011; Stevens et al. 2014). The same colour channel ratio was described in Evans et al. (2010) as the ratio of medium, long and ultra-short wavelength cone types versus the short wavelength type (see **General Methods Chapter**). Hue was not calculated for the white cheek because this is an achromatic ornament. Although hue and saturation colour variables may not necessarily relate to colour perception in birds, avian visual models that incorporate cone sensitivities of the bird's retina and light conditions, have proved to be the most widely approach used to model avian colour vision and colouration (Stoddard and Prum 2008; Kemp et al. 2015).

Statistical analyses

All analyses were performed in R version 3.1.3 (R Development Core Team 2015). Because we were interested in evaluating the effects of several characteristics of the

breeding pair on eggshell pigmentation, partial least squares regression (PLSR) was the most appropriate tool for the analyses. The PLSR is especially useful when the number of predictor variables is similar to or higher than the number of observations and predictors are highly correlated (Carrascal et al. 2009). Indeed, recent studies in bird ecology have shown that it is extremely robust with small sample size and multicollinearity (Galván et al. 2014). In this study, we used 41 females and 22 continuous predictor variables, and an over-parameterized Gaussian GLM model showed high variance inflation factors due to multicollinearity (VIF>5, Table A1). However, the PLSR deals with over-parameterization, and we were able to regress the proportion of eggshell spottiness against the whole set of 22 variables (see Appendix for further detail). The explanatory variables included clutch size, hatching date, and several variables for both members of the social pair: body mass, plumage colour (see results for details on colour variables), and infestation scores for 5 different parasite species. In order to account for the fact that male colour variables included cheek ornamentation (whereas cheek colour was not measured in females), we run additional models with male and female variables separately, and with identical variables for both sexes (i.e. excluding male cheek ornamentation). These models gave similar results (see Appendix and Table A2 for further detail).

Because the mixture of categorical and non-categorical indicators in the PLSR is not recommended (Jakobowicz and Derquenne 2007), we performed two additional PLSR analyses in order to explore the relationship between individual characteristics and age. The model gave similar results for yearlings but was not stable after cross-validation for adult birds, probably due to reduced sample size and presence of outliers (see Appendix and Table A3). For this reason, the data presented here corresponds to the stable model without the categorical age variable. In order to test the interaction effect of male and female age on eggshell pigmentation, we used linear models. For most individuals, the exact age could be estimated using ringing records from the long-term monitoring project established for the study population (Merino et al. 1997). Individuals for which the exact

age could not be assigned were excluded from the analyses (i.e. new recruits from immigrants that were assigned a minimum age of two years). In the 2012 breeding season, the age of the male and female partners was not correlated (t = 1.23, df = 71, Pearson's correlation: r = 0.14, P = 0.22). The initial model included both age and its squared term in order to test linear and quadratic age effects. However, models including both the quadratic terms and the interaction term between female and male age showed high correlation between covariates (as evidenced by VIF>20). Therefore, only the minimal adequate model with linear predictors and main effects is presented.

Finally, we evaluated the relationship between eggshell pigmentation and paternity. We used robust regression analysis based on leverage because the residual plots from a GLM approach revealed patterns due to influential points. Pigmentation scores were used as dependent variable, and to most effectively distinguish the gain or loss of paternity, two variables were used as independent variables: cuckolded and extra-pair paternity. Both variables were converted to a binary trait (yes/no): cuckolded refers to a male that lost paternity with another male from the population and extra-pair paternity refers to a male that gained paternity in a different nest. Effect sizes were calculated as the Cohen's d (Cohen 1998). In this blue tit population, being cuckolded was independent of males having extra-pair nestlings (c^2_1 = 0.33, N = 79, P = 0.56).

After multiple testing on the same data, we used the false discovery rate (Benjamini and Yekutieli 2001) to correct all p-values from the resulting models.

RESULTS

Eggshell pigmentation, colour ornaments and parasitic infections

A total of 41 clutches (see methods) were used in the PLSR analysis. The 22 predictor variables are detailed in Table 1. The PLSR was well calibrated (critical $Q^2>0$ from cross-validation, see Wold et al. 2001), and the linear model revealed only one highly significant latent component ($F_{1,39}$ = 40.79, N = 41, P<0.001) that accounted for 51.1% of the variation in eggshell pigmentation (Table 1). After bootstrapping, the PLSR component accounted for an averaged 59.2% of the variation in eggshell pigmentation, ranging from 42.5 to 75.4% at 95% confidence interval.

The weights for 8 predictor variables were significantly different from 0, indicating that these variables were stable in the PLSR component after bootstrapping (clutch size, female body mass, white cheek saturation in males, yellow breast saturation in males and females, infection by *Leucocytozoon* A in females, and infection by *Haemoproteus* and *Plasmodium* in males, see Fig. 2. Briefly, increased eggshell pigmentation was related to females that were lighter, more saturated in the yellow breast feathers, more intensely infected by *Leucocytozoon* A, and had smaller clutches. The male mates from those nests were in turn more saturated in the white cheek, less saturated in the yellow breast feathers, significantly more intensely infected by *Haemoproteus* and less intensely infected by *Plasmodium*. Thus, 8 predictor variables from females and males were responsible for 34.3% of the variation in eggshell pigmentation (Table 1). Eggshell pigmentation was also explained by male plumage colour and male infestation by several parasite species. Male plumage colour accounted for 14.5% of the variation in eggshell spottiness (breast colour 9.5% and cheek colour 5%, but see additional analyses without cheek colour variables in the Appendix), and male infestation by parasites for another 14.3%. Infection by *Haemoproteus* and *Plasmodium* in males explained 4.2% and 6.1% of the variation

respectively. The prevalence of infection by these parasite species in blue tit males was high: 31.7% for *Plasmodium* and 97.6% for *Haemoproteus*.

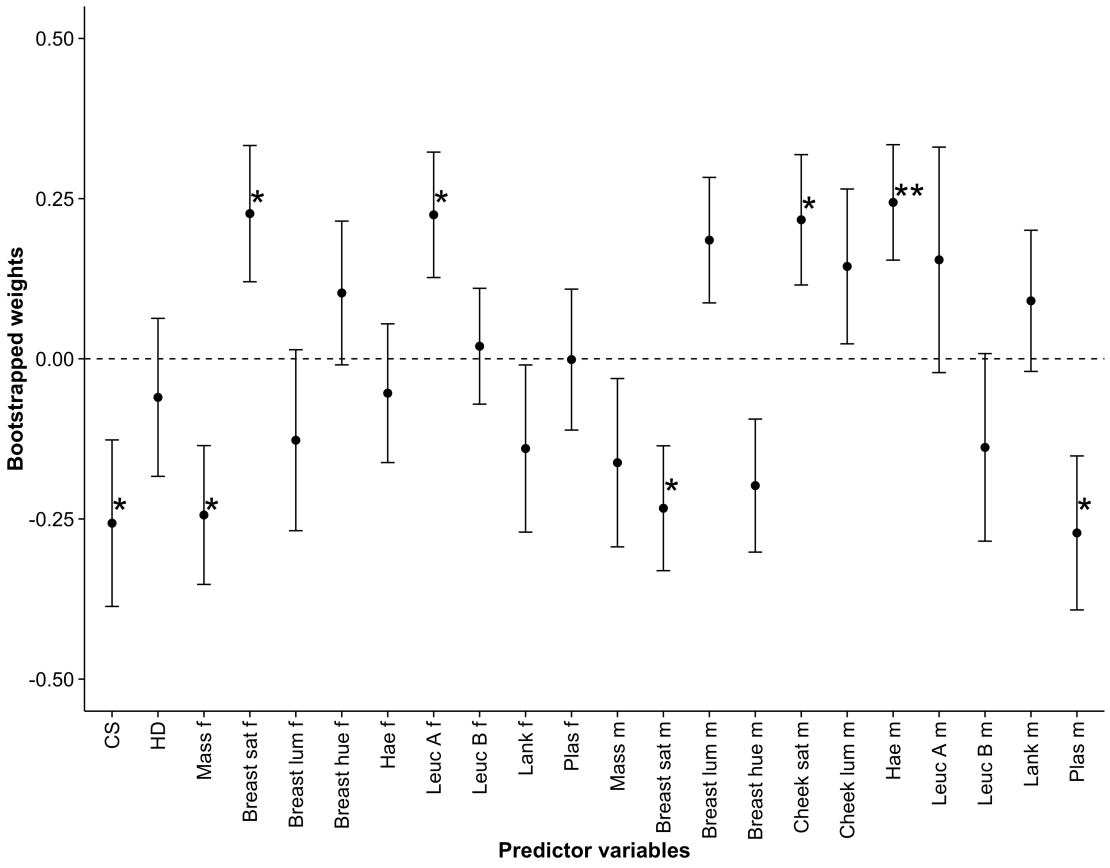

Figure 2. Bootstrapped weights and standard error for each predictor variable included in the PLS regression analysis. Negative weight values indicate a negative relationship with the response variable. When appropriate, significant p-values are shown. Significant coefficients are indicated with an asterisk: *p < 0.05, **p < 0.01. Codes: CS=clutch size, HD=hatching date, f=female, m=male, sat=saturation, lum=luminance, Hae=*Haemoproteus majoris*, Leuc=*Leucocytozoon majoris*, Plas=*Plasmodium* spp., Lank=*Lankesterella valsainiensis.*

Table 1. Results of the partial least squares regression (PLSR) for the spottiness in the eggshell of blue tits' eggs. All predictor variables defining the single and significant ($P < 0.05$) latent component and their weights are shown. Variables that were significant after bootstrapping are in bold type.

Predictor variable	Weight	Contributions by categories of variables (R^2 in %)
Phenological variables		5.2
Clutch size	**-0.311**	**4.9**
Hatching date	-0.077	0.3
Female body mass	**-0.286**	**4.2**
Female plumage colour		5.5
Breast		
saturation	**0.270**	**3.7**
luminance	-0.139	1.0
hue	0.124	0.8
Female infestation by parasites		5.3
Haemoproteus	-0.065	0.2
Plasmodium	-0.003	0.0
***Leucocytozoon* A**	**0.268**	**3.7**
Leucocytozoon B	0.024	0.0
Lankesterella	-0.164	1.4
Male body mass	-0.202	2.1
Male plumage colour		14.5
Breast		9.5
saturation	**-0.283**	**4.1**
luminance	0.220	2.5
hue	-0.238	2.9
Cheek		5.0
saturation	**0.259**	**3.4**
luminance	0.179	1.6
Male infestation by parasites		14.3
Haemoproteus	**0.285**	**4.2**
Plasmodium	**-0.346**	**6.1**
Leucocytozoon A	0.188	1.8
Leucocytozoon B	-0.183	1.7
Lankesterella	0.101	0.5
R^2 by the whole factor (in %)		**51.1**

Eggshell pigmentation and paternity

Overall, 43% of the nests (34/79) contained at least one extra-pair young and 13% (84/607) of all offspring genotyped were sired by a male other than the social father.

Eggshell pigmentation was significantly higher when males had extra-pair nestlings in a different nest ($F_{1,73}$ = 5.52, N = 77, P = 0.022, effect size Cohen's d = 0.6). In nests with more pigmented eggs, males tended to be marginally more cuckolded ($F_{1,73}$ = 3.01, N = 77, P = 0.087, effect size Cohen's d = 0.4).

Eggshell pigmentation and age

We found no significant effect of female age on eggshell maculation ($F_{1,68}$ = 0.15, N = 71, P = 0.7). However, male age was significantly related to eggshell pigmentation ($F_{1,68}$ = 5.52, N = 71, P = 0.022, effect size Cohen's d = 1.89), with older males attending nests with less pigmented eggs (Fig. 3). The likelihood ratio test showed that the model with linear predictors and the interaction between female and male age was not significantly different from the null model (LR test, c^2_2 = 6.1, N = 71, P = 0.11).

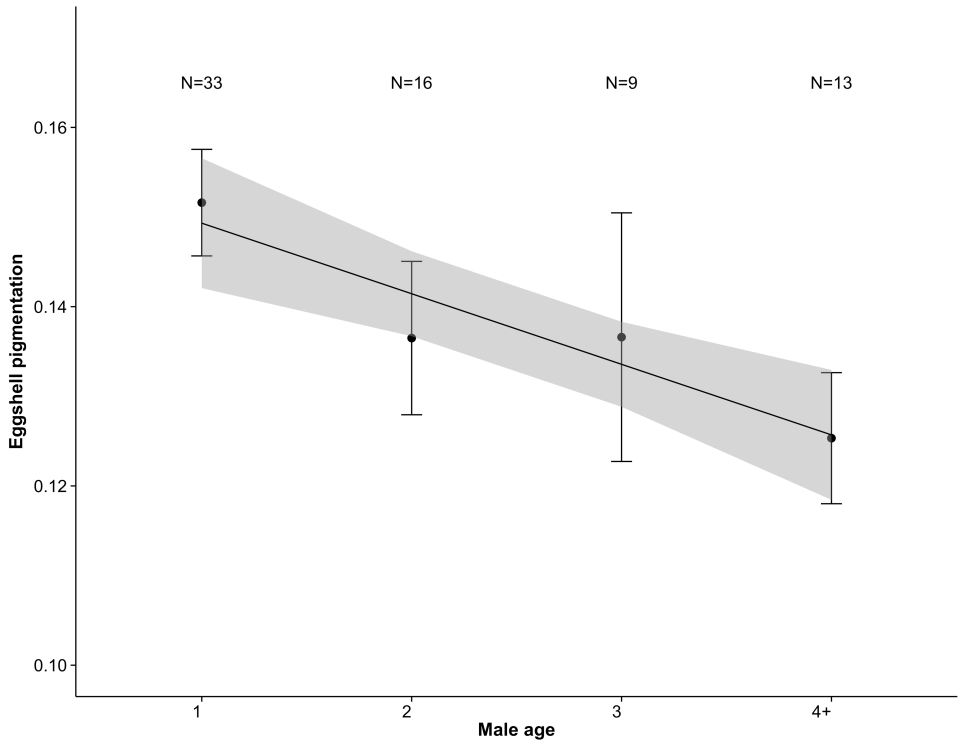

Figure 3. Eggshell pigmentation in relation to male age. Eggshell pigmentation values refer to the proportion of pigments in each egg averaged per clutch (see methods section). Data are shown as mean ± standard error. The predicted regression line ± 95% confidence intervals (shaded area) are also indicated. Sample sizes for each group are shown above the bars

DISCUSSION

In blue tits breeding in central Spain, we found that male characteristics explained most of the variation in eggshell pigmentation, with more pigmented eggs generally having paler and more parasitized fathers. With the use of a robust PLSR approach, we evaluated several characteristics from each individual at the same time. Thus, male blue tits that had higher parasitaemia by the blood parasite *Haemoproteus* (but less by *Plasmodium*) and showed lower saturation in their yellow breast feathers, attended nests with more pigmented eggs. According to these, they were lower quality males. Indeed, previous studies in the present population have shown that the blood parasite *Haemoproteus* exerts negative effects in the bird's performance (Merino et al. 2000; Martínez-de la Puente et al. 2010), whereas the intensity of infections by other parasites like *Plasmodium* is very low and usually remains undetected (del Cerro et al. 2010). Although *Plasmodium* infections are detrimental in other blue tit populations (Lachish et al 2011), we found no significant effect on yellow colour in our population. Chronic infections by *Plasmodium* that show greatly reduced parasitaemia may bear minimal fitness costs of infection (Valkiūnas 2005).

The effect of pathogens like malarial parasites on yellow colouration has been demonstrated in finches (Hill et al. 2004). Carotenoid colouration is thus likely to reflect the negative effects of parasitic infections by *Haemoproteus*. For the breeding season of 2012, we found that more intensely parasitized blue tit males were paler, and that these males paired with females that laid more pigmented eggs. The paler yellow colour that we observed in 2012 was originated during the moult after the 2011 breeding season, but it may still reflect previous infection by *Haemoproteus* because relapses from malaria-like parasites are common during reproduction (Valkiūnas 2005). In fact, bird colouration may signal male quality in terms of the individual's performance in the previous breeding season (Griggio et al. 2009). Data from the intensity of infection in the 2011 breeding

season was not available, so this hypothesis needs further confirmation. In any case, it is possible that highly parasitized males were unable to provide enough extra-food resources to their laying partners due to reduced foraging ability (Marzal et al. 2005), which in turn laid more pigmented eggs. Though this hypothesis is at this stage speculative, it is further confirmed by our results on yellow colouration. Blue tit males with more saturated yellow breast feathers are known to be better foragers (García-Navas et al. 2012).

Interestingly, males that were intensely parasitized by *Haemoproteus* and paler in their breast feathers also tended to have higher saturation values in their white feathers. White from unpigmented feathers is produced by incoherent scattering of all ambient light waves (Prum 2006), which ultimately depends on the feather microstructure. Because saturation refers to the amount of colour compared to white light (Stevens 2011), white feathers should have low saturation values. Therefore, it is possible that higher saturation in the white cheek of the blue tit males also indicated poor condition. Other studies, however, could not find a link between white colouration and condition. Instead, it has been suggested to serve as signal in a different context. For example, patch immaculateness in the white cheek predicted higher social status and reproductive success in a similar species, the great tit (Ferns and Hinsley 2004).

Several non-exclusive hypotheses that we detail further below might be considered to explain male effects on spotting coverage: (i) direct effect of poor/reduced courtship feeding due to the male's poor foraging ability, (ii) other aspects correlated with male condition, for example its territory, or (iii) differential allocation from females according to male ornamentation (Sheldon 2000). First, pigment deposition in the eggshell may be affected by the male's ability to feed the female. The male blue tit feeds the female off the nest prior to laying, but because it is hard to detect in the field, this behaviour is often ignored (Royama 1966). In fact, the extra-food resources required to lay the whole

clutch may be over 150% the female's weight in small birds like the blue tit (Perrins 1970), and a recent study supports the evolution of mate-feeding to compensate for the nutritional limitation of females during the entire breeding period (see Galván and Sanz 2011, and references therein). Thus the food offered by the male prior to laying is likely to influence the female's condition and potentially eggshell spottiness. Our results support this, because female blue tits laying more pigmented eggs were likely to be in poor condition. This was shown by higher intensity of infection by the blood parasite, *Leucocytozoon* A, reduced body mass, and smaller clutches (but they were also more saturated in the yellow breast, discussed below). Besides, males could assess female quality using egg colour (Moreno and Osorno 2003) and female ornamentation, and thus adjust courtship feeding behaviour. Experimental confirmation of this is lacking, but changes in male provisioning rates to nestlings in response to female characteristics have been reported in other species (Soler et al. 2008; English and Montgomerie 2011).

The second hypothesis to explain male effects on spotting coverage is that the increased eggshell pigmentation could be a by-product of the female's mate choice. When paired with lower-quality males, females may lay more spotted eggs because they are left with less food resources in the pair's breeding territory. Certainly, more spotted eggs are common in lower quality habitats (reviewed in Gosler et al. 2005). Our results on male age and eggshell pigmentation are in agreement with this, because younger males attended nests with more spotted eggs; and younger blue tit males in other European populations usually nest in lower quality habitats (Amininasab et al. 2016). Furthermore, this hypothesis could also explain why we found a negative relationship between female colouration in the yellow patch and female condition. Females that laid more pigmented clutches were more saturated in their breast feathers. The yellow colouration might reflect the bird's performance in the previous breeding season (see above), and thus the reduced body mass in these females could just be a result of breeding in a poor territory in the present season. However, these females were also more intensely parasitized by a

lineage of the parasite *Leucocytozoon*, and it may be that the effects of haematozoan parasites vary within breeding season, region and bird species (Hill 2006a), or even between sexes. Thus, we remain cautious as to how we interpret this relationship in females. More experimental work is needed to understand accurately the link between parasitism and signalling for carotenoid colouration in females in our blue tit population. For example, including female cheek colour in future studies might reveal a clearer association between ornamentation and parasitism in relation to eggshell pigmentation.

Finally, we propose a third hypothesis to explain the relationship between male characteristics and eggshell spottiness. Female birds can adjust their investment in a particular reproductive event depending, for instance, on male colouration (Giraudeau et al. 2011). Indeed, there is some evidence that females change clutch size depending on male ornaments (Velando et al. 2006). Our results showed that more pigmented clutches from low quality fathers were significantly smaller, which is in agreement with previous findings in female great tits (Hargitai et al. 2016). Females may also compensate for mating with males in poorer condition by allocating more pigments to the eggshell if for instance, this confers eggshell strength under calcium deficiency in poor breeding territories (Gosler et al. 2005). Furthermore, more protoporphyrin-pigmented eggshells may also contain more biliverdin (Wang et al. 2009; Duval et al. 2013b). Biliverdin is an antioxidant pigment that might be traded off against female antioxidant capacity (Moreno and Osorno 2003), and together with protoporphyrin, has been shown to be responsible for background eggshell colour (Gorchein et al. 2009). Unfortunately, biliverdin concentration in the eggshell could not be assessed in this study because blue tit eggs were not collected for ethical reasons. Nonetheless, experimental evidence that a change in eggshell pigmentation/background is a direct response to male quality in blue tits is lacking.

According to our results on female condition, parasitic infections and extra-pair paternity we suggest that poor-quality females laid more spotted eggs. However, two alternative hypotheses have been proposed to explain why females lay more brown-pigmented eggs (Moreno and Osorno 2003): (i) high quality females that remove the damaging pigments from blood efficiently and deposit them in the eggshell, or (ii) females in low condition that suffer from higher protoporphyrin levels in blood and are unable to remove them from their system, resulting in increased eggshell pigmentation because of the protoporphyrin excess. Previous studies in blue tits offer support for the first hypothesis: high quality females might lay eggs with less distributed spots, concentrated in one end of the egg forming a 'corona' ring (Sanz and García-Navas 2009; García-Navas et al. 2011; Holveck et al. 2012). On the contrary, Martínez-de la Puente et al. (2007) found, in our study population, that lower quality females had increased levels of stress proteins, produced more spotted eggs and paired with lower quality males. Although in great tits, Hargitai et al. (2016) also found that poor-quality females laid darker and more pigmented eggs. Female blue tits in our study population could be suffering carry-over effects from poor condition during the non-breeding season (Robb et al. 2008; Harrison et al. 2011). Our findings concerning extra pair paternity agree with the fact that females laying more pigmented eggs were in poor condition, because these clutches tended to have unfaithful fathers. Extra-pair copulations in blue tits are frequent during the females' fertile period; this is, during egg laying (Kempenaers et al. 1995). Male blue tits could seek extra-pair copulations precisely because they are paired with low quality females. This is in agreement with studies in other bird species that mated in low quality environments (O'Brien and Dawson 2011; Yuta and Koizumi 2016). Another possibility is that increased eggshell pigmentation signalled that females were in poor condition, and thus males compensated for this by seeking extra-pair offspring. However, there is mixed support for the role of eggshell colour as sexually selected signals in cavity nesting birds (Cherry and Gosler 2010; Reynolds et al. 2009), for which dim conditions inside the nestbox were

thought to make it difficult to discriminate by colour (Cassey 2009; Duval et al. 2013a). Support for the ability to discriminate eggshell colour comes from recent avian visual models (Holveck et al 2010; Gomez et al. 2014), but experimental evidence that this could be the case in wild blue tits is lacking. In addition to this, in our study, males attending more pigmented clutches tended to be marginally more cuckolded. This could be explained if males that engaged in extra-pair copulations unattended guarding activities in their social nest (Garcia-Navas et al. 2014); or, if females sought extra-pair mates because their social male was also in poor condition. Some studies suggest that females can mate socially with low quality males but seek extra-pair mates (Arct et al. 2015); but we must be cautious when interpreting this result because it is not clear whether the costs of extra-pair mating to females are higher than the benefits (Vedder et al. 2011; Forstmeier et al. 2014). In fact, breeding pairs with no cuckoldry (from females) and no extra-pair paternity (from males) might be of higher quality. This is partially confirmed by the fact that egg pigmentation was marginally lower in those nests (Kruskal-Wallis test c^2_3 = 7.4756, N = 77, P = 0.058), but further experiments are needed.

In conclusion, we showed that parasitic infections and ornamentation in both members of the breeding pair may be good indicators of eggshell quality, which in turn might have important implications for embryo development. We have also discussed how the male's age and extra-pair paternity may be related to eggshell pigmentation. Male blue tits' characteristics might be relevant to females during egg formation. The male's courtship feeding behaviour may have an effect on female condition and pigment deposition on the eggshell. Alternatively, females pairing with lower quality males might deposit more protoporphyrin to the eggshell as a result of breeding in lower quality territories, or they may adjust pigment deposition in response to lower quality males, but these hypotheses require further confirmation.

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APPENDIX

Statistical modeling

PLSR analysis is an extension of the multiple regression analysis based on a linear conversion from a large number of original descriptors to a small number of orthogonal factors (or latent components), which are related to the response variable by ordinary least squares regression (Abdi 2010). The interpretation of latent components was derived from the weights and loadings of original variables, and the relative contribution of each variable was calculated by means of the square of predictor weights (Abdi 2010). The predictability of the results was tested by means of a cross-validation procedure with a data-splitting strategy by which we built PLSR models with two-thirds of the blocks randomly selected from the original sample, and predicted the eggshell pigmentation in the remaining one-third of the sample. After model checking we found one influential point, and thus the averaged weights after bootstrapping with 2,000 samples are presented.

Additional PLSR models on males and females

In order to explore whether the same conclusions could be drawn from a model that only included male or female characteristics alternatively, we performed additional PLSR models. The PLSR model that only included female variables (clutch size, hatching date, body mass, yellow colour variables, and intensity of the infection by the 5 parasite species) was not stable as shown by cross validation (very low critical Q^2). However, two models that include male variables were validated and stable through cross validations. The first PLSR model included all male variables (body mass, white and yellow colour variables, and intensity of the infection by the 5 parasite species) plus clutch size and hatching date and explained 46.12% of the variation in spottiness coverage (results not shown). The second PLSR model included male variables with the exception of white

cheek colour (body mass, yellow colour variables, and intensity of the infection by the 5 parasite species) plus clutch size and hatching date. It explained 39.65% of the variation in spottiness coverage. Both models gave similar results to those shown in the main text.

Finally we performed a PLSR model that included female and male characteristics except male cheek colour variables. This model confirmed that male colour variables explained more variation in eggshell spottiness than female colour variables when the same male and female colour variables were evaluated (9.6% for male vs. 5.6% for female yellow colour, Table A2).

PLS Regression model on yearlings

A total of 22 clutches were used in an additional PLSR analysis on yearlings. We used the same 22 predictor variables as detailed in the main text. The PLSR was well calibrated (critical $Q^2>0$ from cross-validation, see the main text), and the linear model revealed only one highly significant latent component ($F_{1,20} = 41.43$, $N = 22$, $P<0.001$) that accounted for 67.4% of the variation in eggshell pigmentation (Table A3). After bootstrapping, the PLSR component accounted for an averaged 74.3% of the variation in eggshell pigmentation, ranging from 58.6 to 88.7% at 95% confidence interval.

The weights for 5 predictor variables were significantly different from 0, indicating that these variables were stable in the PLSR component after bootstrapping (clutch size P=0.05, yellow breast saturation in males P=0.02 and in females P=0.04, and infection by *Haemoproteus* P=0.03 and *Plasmodium* P=0.01 in males). The relationship with eggshell pigmentation and male and female characteristics in yearlings are the same as the ones detailed in the main model in the text. Briefly, increased eggshell pigmentation was related to females that had smaller clutches and were more saturated in the yellow breast feathers. Yearling male mates from those nests were in turn less saturated in the yellow breast feathers, significantly more intensely infected by *Haemoproteus* and less intensely

infected by *Plasmodium*. Particular contributions for each variable are detailed in Table A3.

We also designed a model with older individuals (N=19) but this model was not supported by cross-validation (the critical Q^2 was not sufficient to ensure that the model was well calibrated). This might be due to reduced sample size and presence of outliers.

Tables

Table A1. Variance Inflation Factors (VIF) for the GLM with the set of 22 variables. VIF>5 (shown in bold) indicate that standard errors are doubled for those variables.

Predictor variable	VIF
Clutch size	2.319150
Hatching date	2.000102
Female mass	2.773376
Female breast saturation	6.825775
Female breast luminance	3.695251
Female breast hue	7.939241
Female infection by *Haemoproteus*	1.584660
Female infection by *Leucocytozoon* A	2.478513
Female infection by *Leucocytozoon* B	3.292679
Female infection by *Lankesterella*	2.628262
Female infection by *Plasmodium*	1.745950
Male mass	2.012152
Male breast saturation	6.744476
Male breast luminance	2.830038
Male breast hue	6.777308
Male cheek saturation	2.076444
Male cheek luminance	2.976065
Male infection by *Haemoproteus*	1.439271
Male infection by *Leucocytozoon* A	2.353154
Male infection by *Leucocytozoon* B	2.021605
Male infection by *Lankesterella*	2.009813
Male infection by *Plasmodium*	2.217069

Table A2. Results of the partial least squares regression (PLSR) for females and males without cheek colour variables. All predictor variables defining the single and significant ($P < 0.05$) latent component and their weights are shown. Variables that were significant after bootstrapping are in bold type.

Predictor variable	**Weight**	**Contributions by categories of variables (R^2 in %)**
Phenological variables		5.3
Clutch size	-0.11	5.0
Hatching date	-0.01	0.3
Female body mass	**-0.09**	**4.3**
Female plumage colour		5.6
Breast		
saturation	**0.08**	**3.8**
luminance	-0.02	1.0
hue	0.02	0.8
Female infestation by parasites		5.6
Haemoproteus	-0.046	0.2
Plasmodium	-0.000	0.0
***Leucocytozoon* A**	**0.08**	**3.7**
Leucocytozoon B	0.001	0.3
Lankesterella	-0.03	1.4
Male body mass	-0.05	2.1
Male plumage colour		9.6
Breast		
saturation	**-0.09**	**4.2**
luminance	0.165	2.5
hue	**-0.213**	**2.9**
Male infestation by parasites		14.4
Haemoproteus	**0.249**	**4.2**
Plasmodium	**-0.407**	**6.2**
Leucocytozoon A	0.312	1.8
Leucocytozoon B	-0.33	1.7
Lankesterella	0.099	0.5
R^2 by the whole factor (in %)		**46.9**

Table A3. Results of the partial least squares regression (PLSR) for yearlings. All predictor variables defining the single and significant (*P < 0.05*) latent component and their weights are shown. Variables that were significant after bootstrapping are in bold type.

Predictor variable	**Weight**	**Contributions by categories of variables (R^2 in %)**
Phenological variables		9.9
Clutch size	**-0.28**	**5.3**
Hatching date	-0.262	4.6
Female body mass	-0.207	2.9
Female plumage colour		5.7
Breast		
saturation	**0.24**	**3.9**
luminance	-0.102	0.7
hue	0.123	1.1
Female infestation by parasites		4.8
Haemoproteus	-0.046	0.2
Plasmodium	-0.007	0.0
Leucocytozoon A	0.256	4.4
Leucocytozoon B	0.039	0.1
Lankesterella	-0.038	0.1
Male body mass	-0.216	3.1
Male plumage colour		11.1
Breast		9.4
saturation	**-0.26**	**4.5**
luminance	0.165	1.8
hue	-0.213	3.1
Cheek		1.7
saturation	0.139	1.3
luminance	0.08	0.4
Male infestation by parasites		29.9
Haemoproteus	**0.249**	**4.2**
Plasmodium	**-0.407**	**11.1**
Leucocytozoon A	0.312	6.6
Leucocytozoon B	-0.33	7.4
Lankesterella	0.099	0.6
R^2 by the whole factor (in %)		**67.4**

Appendix Figures

Figure A1. Breast reflectance spectra for the blue tit (*Cyanistes caeruleus*) in the 2012 breeding season. Bars denote 95% confidence interval. a) males (N= 41) b) females (N=41).

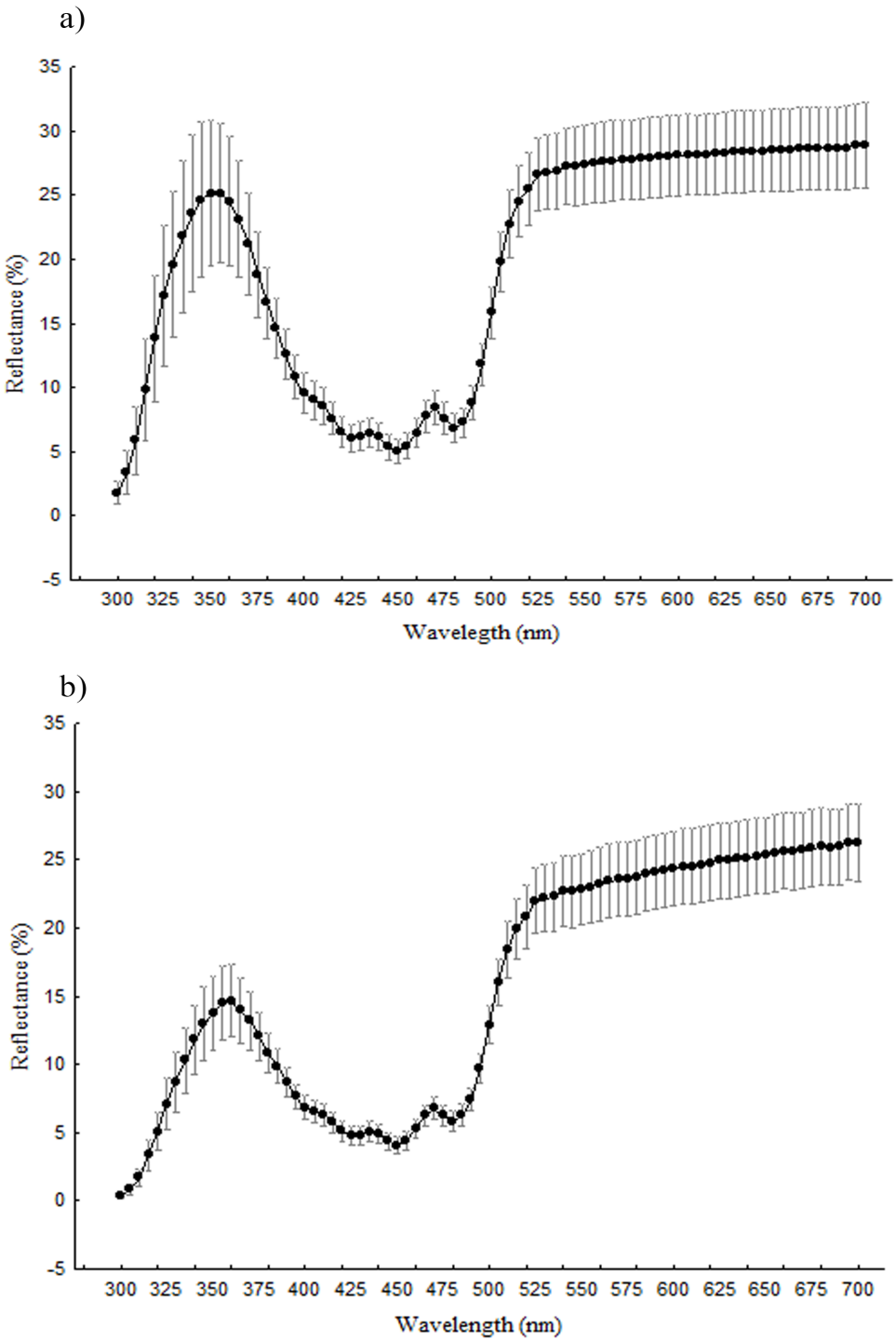
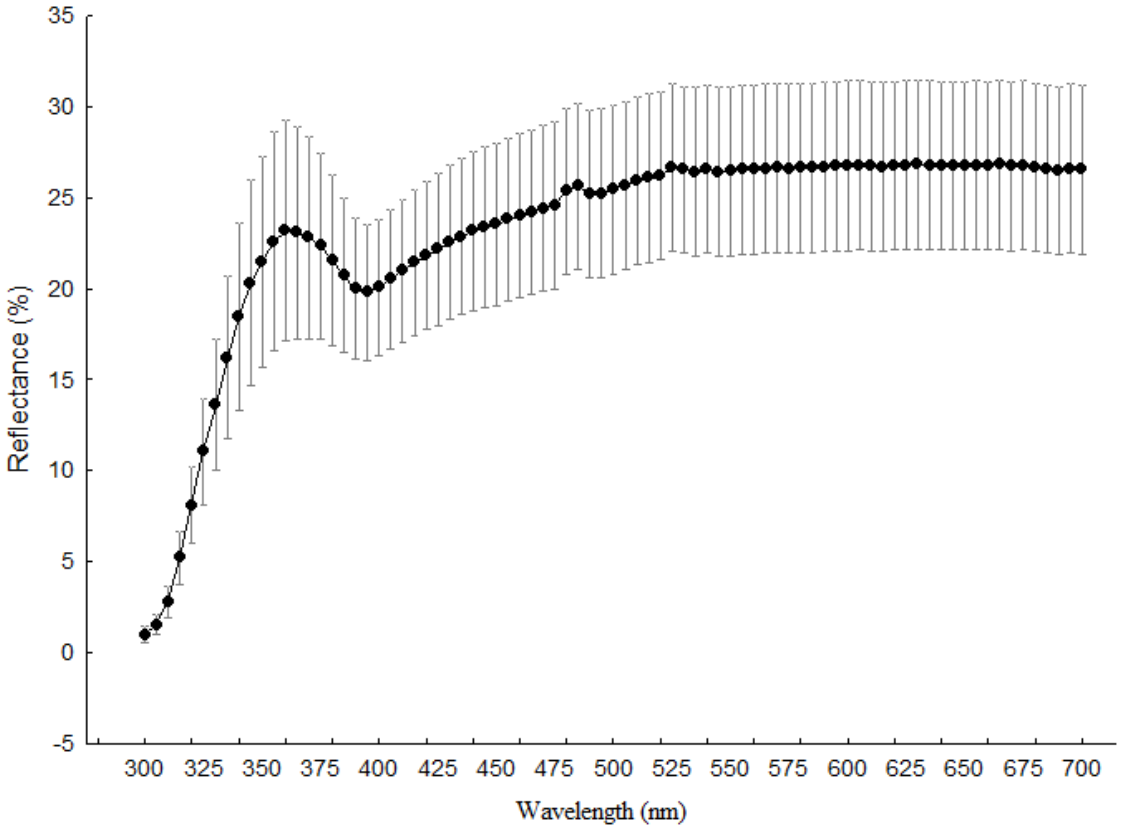


Figure A2. Cheek reflectance spectra for male blue tits (*Cyanistes caeruleus*) in the 2012 breeding season (N_{males}= 41). Bars denote 95% confidence interval.



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Chapter 4

'It is not often that a man can make opportunities for himself. But he can put himself in such shape that when or if the opportunities come he is ready.'

—Theodore Roosevelt

This chapter reproduces entirely the manuscript:

E.P. Badás, A. Autor, J. Martínez, J. Rivero-de Aguilar, and S. Merino. Paternity gain in older male blue tits is related to carotenoid-based colouration, blood parasite infections and colour change between breeding seasons.

Paternity gain in older male blue tits is related to carotenoid-based colouration, blood parasite infections and colour change between breeding seasons

E.P. BADÁS, A. AUTOR, J. MARTÍNEZ, J. RIVERO-DE AGUILAR B, AND S. MERINO

Abstract Extra-pair paternity is a widespread reproductive behaviour among birds. It has been hypothesized that females may seek extra-pair copulations with high quality males, but findings regarding male ornamentation and paternity gain are controversial. This study examines the relationship between male quality and mating strategies in a passerine bird, the blue tit (*Cyanistes caeruleus*). Adult male blue tits have several highly variable carotenoid and structural-based plumage patches on their crown, breast, cheek and tail feathers; and in a population breeding in nest-boxes in central Spain, individuals are usually infected by several blood parasite species. We tested the prediction that males in better condition, more ornamented and less intensely infected have higher extra-pair and total reproductive success. We also measured feather colouration the following breeding season to determine whether changes in ornamentation were related to paternity gain during the previous reproductive event. Older males that (i) had more saturated yellow breasts, (ii) were in better condition, and (iii) harboured higher parasite loads of *Lankesterella valsainiensis*, were more likely to sire extra-pair offspring. Between seasons, we found that these males changed less their feather colouration between seasons. We discuss these results within the context of vector exposure due to engagement in extra-pair copulations and increased likelihood of direct contact with infected individuals. In addition, because the male's feather colouration obtained for the subsequent breeding season was not compromised, we suggest that high-quality males may be able to bear the costs of this mating strategy and benefit from higher reproductive success.

Keywords avian malaria, carry-over effects, coccidians, extra-pair offspring, haemoglobin

INTRODUCTION

Among socially monogamous bird species, extra-pair matings constitute a relatively common strategy (Griffith et al. 2002; Westneat and Stewart 2003). Fitness might then depend on both within- and extra-pair success, and in males, both polygyny and extra-pair paternity have proved to increase variance in reproductive success (Vedder et al. 2011). Thus, avian extra-pair mating systems serve as excellent models to study female choice for higher quality males because in some species such as the blue tit (*Cyanistes caeruleus*) females actively seek extra-pair copulations (Kempenaers et al. 1992). Conflicting results have been reported with respect to male quality and paternity in this species. For example, Johannessen et al. (2005), found no effect on paternity loss after experimentally reducing the males' dominance rank in individuals that were thus perceived as low-quality males by females. Additionally, Delhey et al. (2003) hypothesized that male blue tits that were more ornamented in their blue crown feathers maximized within-pair success instead of fathering more extra-pair offspring. However, a growing body of research has shown that higher quality males in this species are more likely to sire extra-pair offspring. Early and longer singing patterns (Kempenaers et al. 1997; Poesel et al. 2006), resistance to parasitic infections (Podmokła et al. 2015), increased survival (Kempenaers et al. 1997), older age (Kempenaers et al. 1997), heterozygosity (Foerster et al. 2006), or increased UV-ornamentation in the blue crown in yearlings (Peters et al. 2006), have been used to explain the gain of paternity in male blue tits.

Indeed, many individual quality variables may explain extra-pair paternity in blue tit males but, in particular, the links between ornamentation and paternity are far from clear. For example, increased yellow and blue feather colouration in male blue tits has been commonly related to higher quality (Sheldon et al. 1999; Senar et al. 2002; Galván 2011; García-Navas et al. 2012) but the effect of ornaments such as the blue tit crown on male extra-pair success is inconclusive (Delhey et al. 2006b). Similar patterns found in

other species and ornaments continue to intrigue ecologists. In the American redstart, for instance, males that were less saturated in their carotenoid-based ornament sired more extra-pair young (Kappes et al. 2009). In another study, carotenoid-supplemented great tit males were less likely to have extra-pair nestlings in their social brood (Helfenstein et al. 2008), which may indicate that more efficient foragers with increased yellow breast colouration lose less paternity. Furthermore, the relationship between paternity gain and colouration in multiple male ornaments is understudied. A recent study in the yellow warbler (*Stetophaga petechia*) showed that paternity patterns in relation to two pigment types in both sexes might be more complex than expected (Grunst and Grunst 2014). Male warblers with high melanin coverage but duller in their carotenoid colouration fathered more extra-pair offspring but lost within-pair paternity, whereas breeding pairs that paired assortatively with respect to colouration in both pigments maximized within-pair success only. It seems clear that further studies evaluating the interactions between signals and male gain of paternity are needed.

The male's performance during the breeding season may be mirrored in ultraviolet plumage characteristics obtained after the moult (Griggio et al. 2009). In many passerines breeding in temperate regions, the moult is constrained to the time immediately after the reproductive event and before moving to wintering grounds (Holmgren and Hedenström 1995). It has been shown that low-quality blue tits moult faster and develop less bright and less saturated blue crown feathers (Griggio et al. 2009), and carry-over effects from reproduction may affect feather colour, at least in the achromatic white cheek (**Chapter 1**). High-quality males engaging in extra-pair copulations may bear the costs of seeking females and still develop ornamented feather colours. Contrarily, males that invest in mate guarding and maximize within-pair success could risk ornamentation because guarding activities may be costly (Birkhead and Møller 1992). If any, the relationship between paternity during a single reproductive event and feather colour achieved after the moult remains unknown. This may have important consequences for the following season,

because the plumage colour achieved after reproduction is maintained throughout the subsequent reproductive period (Nilsson and Svensson 1996).

Physiological parameters that serve as indicators for the individual's body condition may also be related to extra-pair paternity. For example, haemoglobin levels in blood have been positively related to survival (Bańbura et al. 2007), and health (Słomczyński et al. 2006) in nestling blue tits; and thus it is used as a proxy for condition (for a review, Minias 2015). In adult blue tits, the relationship between paternity and haemoglobin concentration remains, however, unstudied. Infection status has recently been related to paternity in the blue tit (Podmokła et al. 2015), but studies combining blood parasite infections and health or condition indicators are absent in the literature. Moreover, studies on the association between feather colouration in multiple ornaments and paternity are lacking, when, in fact, paternity may enhance the opportunity for sexual selection in this species (Vedder et al. 2011).

In this study, we aimed at investigating which individual quality variables explain most of the variation in male paternity in the blue tit (*Cyanistes caeruleus*). In order to do this, we sampled blue tit males to record colour data from four ornaments (yellow breast, blue crown, white cheek and blue-green tail), infections from blood parasites, condition (i.e. total haemoglobin in blood and body mass) and phenological variables from the males' social nest (clutch size and hatching date). We expect higher quality males to be more likely to sire extra-pair offspring, harbour less blood parasites and be more ornamented. In addition to this, we further investigated discriminability of colour change between seasons. For a subsample of individuals, data on paternity from one breeding season (spring of 2013) and colour change from two consecutive seasons was available (springs of 2013-2014). For each plumage patch we aimed at exploring whether polygynous males developed similar feather colour after the moult, because higher quality individuals may have their colouration less affected by the costs of a reproductive event

(Doutrelant et al. 2012). Alternatively, fathering extra-pair young may increase the males' metabolic costs during the breeding season and negatively affect feather colouration obtained after the moult.

METHODS

Study site and sampling

Our study was carried out during the springs of 2013 and 2014 in a Pyrenean Oak *Quercus pyrenaica* forest located in central Spain (Segovia, 40°54'N, 4°01'W, 1200 m.a.s.l.). Long-term studies of breeding activities have been on-going in the present blue tit population since 1991 (Sanz et al. 2003). In the 2013 and 2014 breeding season male and female adult birds were captured at their nestbox while provisioning 3 days old nestlings. Unringed birds were individually marked with a numbered aluminium leg-ring and sex was assigned based on standard plumage characteristics (Svensson 1992). First-years were identified by possession of distinctive, non-adult greater wing coverts (Svensson 1992).

During the spring of 2013 adult and nestling birds were ringed if necessary, weighed to nearest 0.01 g with a digital balance, and tarsus length and time of the day were recorded in order to calculate the corrected body mass index (following Senar 2002). We also took a drop of blood from the brachial vein in all birds, which was collected in heparinized microcapillaries and later used for paternity analyses and parasite quantification in adults. We detected and quantified several parasite species: *Haemoproteus majoris*, *Plasmodium* sp. haplotype cyan1, *Leucocytozoon majoris* haplotypes leuA, leuA1 and leuB, and *Lankesterella valsainiensis* (see Badás et al. 2015 for details on the relative quantitative PCR and primers used). In 2013, another drop of blood from adult blue tits was used to determine haemoglobin concentration in the field using a portable HemoCue Hb 201+ photometer (HemoCue AB, Ängelholm, Sweden), following

(Burness et al. 2001). The haemoglobinometer is a non-invasive and reliable method that has high sensitivity and specificity (in humans, 0.85 and 0.94 respectively, Mills and Meadows 1989).

During the springs of 2013 and 2014 data on feather colouration was collected using a portable spectrophotometer (Ocean Optics Inc., Dunedin, FL, USA) connected to a Pulsed Xenon Light Source (Jaz-PX lamp) (see Badás et al. 2017-**Chapter 3** and **General Methods Chapter** for more details on how measurements were taken). Feather colour reflectance was measured for the crown, breast, cheek and tail patches in adult breeding birds; and relative quantum photon catches were obtained to build models of blue tit vision (Endler and Mielke 2005; Stevens et al. 2009). From this, three variables describing colour were obtained for each colour patch (hue, saturation and luminance), because these is the most common approach used to model avian colour vision and colouration in recent ecological studies (Stoddard and Prum 2008; Kemp et al. 2015) (see **Chapter 1** for further details).

Paternity analyses

Parents and nestlings were genotyped for 8 microsatellite loci; information on microsatellites, primers and polymerase chain reaction (PCR) conditions are detailed in (Badás et al. 2017-**Chapter 3** and **General Methods Chapter**). Allele lengths were determined with the Genemapper 4.0 software. The offspring was assigned as extra-pair if there were at least two mismatches between the genotype of the social father and offspring. Extra-pair paternity (EPP) for a male different than the social male was assigned when one of the sampled males matched all of the offspring's paternal alleles. Paternity was assigned for 73% of all identified extra-pair fledglings (N=98) in the 2013 breeding season using Cervus 3.0 (Kalinowski et al. 2007). Maternity of the social female was confirmed for all nestlings. The mean exclusion probability of the eight markers was

calculated to be 0.99968 for the first (female) parent and 0.99999 for the second (male) parent (given the genotype of the first parent).

Statistical analyses

All analyses were performed in R v.3.1.3 (R Foundation for Statistical Computing, Vienna). First, we explored whether the male's gain of paternity was related to feather colour, blood parasite infections and breeding parameters from the 2013 breeding season. In order to do this, and to avoid multicollinearity or sample size problems in analyses with a high number of explanatory variables, we designed a partial least squares regression model (PLSR) (Carrascal et al. 2009). The PLSR allowed us to include highly correlated colour variables and thus extract the most relevant variables explaining variation in the dependent variable. This method is becoming increasingly popular in ecological studies because it is extremely robust in such cases (Galván et al. 2014; Badás et al 2017 submitted). Here, we used 20 explanatory variables that included cheek, crown, tail and breast colour variables, infection intensity by five parasite species, hatching date and clutch size in the male's social nest, and body mass; and related these to a binomial (yes/no) variable that recorded the male's extra-pair paternity (see Badás et al 2017 submitted for more details on the analyses). The model was fitted using the R package 'plsRglm' (Bertrand et al. 2014) for binomial PLSR, and data on all 20 variables were available for 44 males from the 2013 breeding season. The most parsimonious binomial PLSR was selected using the difference in AIC (Akaike Information Criterion, Akaike 1973) and it revealed that only one component explained most of the variation in extra-pair paternity (see Results section). Finally, the PLSR model weights were averaged after bootstrapping with 2,000 samples, in order to obtain stable variables that explained male paternity in the present blue tit population. PLSR does not allow for missing cells (complete data on 20 variables was available for 44 males). However, complete data on feather colouration was available for 61 males. Therefore, in order to confirm our results

on paternity and colouration we performed an additional model that included saturation and luminance for all three patches (cheek, crown, and breast) and tail luminance (Generalized Linear Model GLM, robust regression with binomial error distribution). Breast and crown hue could not be included in the model because they were highly correlated to saturation in each patch (all P-value <0.0001, r >0.85); tail saturation was highly correlated to crown saturation, and it was also dropped from the model (t=12.06, df=59, P-value <0.0001, r=0.84, N=63).

From the 2013 breeding season, 19 males were recaptured in the 2014 breeding season. Using data on colour from both breeding seasons, we extracted JND scores, or Just Noticeable Differences (Siddiqi et al. 2004), which described colour change between seasons. We performed two-sample t-tests (or paired sign tests when the assumptions of normality were violated) relating chromatic or achromatic just-noticeable differences (JNDs) to male paternity, which was codified as a binary trait (yes/no). We also evaluated whether male reproductive success (total number of fledglings) in 2013 was related to detectable changes in plumage through JND values and linear regression models. Effect sizes were calculated by means of Cohen's D (Cohen 1998) or the non-parametric version when the differences between group variances were high (Cliff's Delta, R package 'effsize', Torchiano 2016).

RESULTS

A total of 559 nestlings and 164 adults from 89 breeding pairs were genotyped for paternity. We successfully assigned paternity to 70% (69/98) of extra-pair offspring. Overall, more than half of the nests (55%, 43/78) contained at least one extra-pair young and 19% (98/522) of all offspring genotyped were sired by a male other than the social father. Nestlings from eleven breeding pairs could not be genotyped because of nest desertion (see **Chapter 2**), and thus, these males were not included in the analyses.

Within the 2013 breeding season, extra-pair paternity was significantly different with age (χ^2_1=12.65, p-value=0.00038, N=61); in fact, no yearlings had extra-pair nestlings. Male extra-pair paternity was explained by a single PLSR component that accounted for 53.08% of the variation in extra-pair paternity (Table 1, N=44). Males that had extra-pair nestlings outside the social nest were more saturated and showed higher values of hue in their yellow breast feathers, they were also more intensely infected by the blood parasite *Lankesterella valsainiensis* and had higher haemoglobin levels in blood (Table 1, Fig 1). An additional binomial GLM model with feather colour variables only confirmed that male extra-pair paternity was positively related to breast (z=2.11, P-value=0.035, N=61) and crown (z=1.99, P-value=0.0472, N=61) saturation, and that it tended to be positively related to tail luminance (z=1.94, P-value=0.0528, N=61).

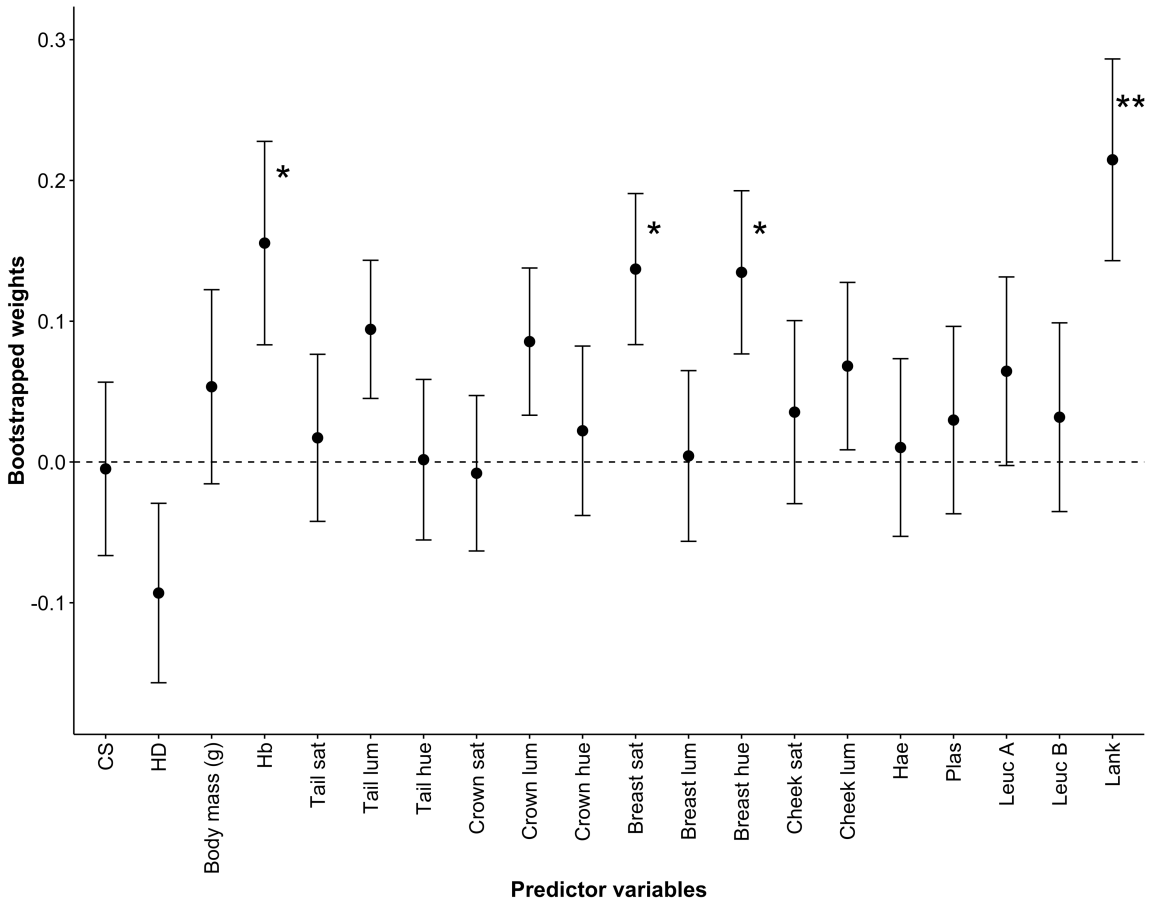

Figure 1. Binomial PLS regression on variables explaining extra-pair paternity in male blue tits. Shown are bootstrapped weights and standard error for each predictor variable included in the model. Negative weight values indicate a negative relationship with the response variable. When appropriate, significant p-values are shown. Significant coefficients are indicated with an asterisk: *p < 0.05, **p < 0.01. Codes: CS= clutch size, HD= hatching date, Hb= haemoglobin concentration, sat= saturation, lum= luminance, Hae= *Haemoproteus majoris*, Plas= *Plasmodium* spp., Leuc= *Leucocytozoon* spp., Lank= *Lankesterella valsainiensis*.

Table 1. Results of the partial least squares regression (PLSR) for extra-pair paternity of male blue tits. All predictor variables defining the single latent component and their weights are shown. Variables that were significant after bootstrapping are in bold type.

Predictor variable	**Weight**	**Contributions by categories of variables (R^2 in %)**
Phenological variables		2.34
Clutch size	-0.013	0.01
Hatching date	-0.210	2.33
Condition		7.86
Body mass	0.105	0.59
Haemoglobin	**0.370**	**7.27**
Plumage colour		17.93
Breast		11.38
saturation	**0.332**	**5.84**
luminance	0.029	0.04
hue	**0.322**	**5.50**
Cheek		1.65
saturation	0.071	0.27
luminance	0.161	1.38
Crown		2.3
saturation	-0.031	0.05
luminance	0.206	2.25
hue	-0.08	0.00
Tail		2.6
saturation	0.022	0.03
luminance	0.218	2.51
hue	0.035	0.06
Infestation by parasites		24.94
Haemoproteus	0.024	0.03
Plasmodium	0.072	0.27
Leucocytozoon A	0.123	0.84
Leucocytozoon B	0.078	0.32
Lankesterella	**0.665**	**23.48**
R^2 by the whole factor (in %)		**53.1**

When we explored male colour differences between seasons, we found that visual chromatic contrasts in their breast feathers were related to extra-pair paternity (t=2.91, df=13, p-value=0.013, N=19, effect size ES=0.69, Fig. 2a) and number of nestlings ($F_{1,16}$=5.54, p-value=0.03, effect size ES=1.18). In other words, when males had extra-pair nestlings and higher reproductive success during the 2013-breeding season, their yellow breast colour changed less between seasons (2013 vs. 2014). Extra-pair paternity was also

negatively related to the achromatic contrasts in the white cheek (t=2.42, df=17, p-value=0.03, N=19, effect size ES=0.59, Fig. 2b) and in the blue-green tail (t=2.48, df=12, p-value=0.03, N=18, effect size ES=0.66, Fig. 2c). Visual (chromatic and achromatic) contrasts for the blue crown were not related to paternity (all p-value>0.05).

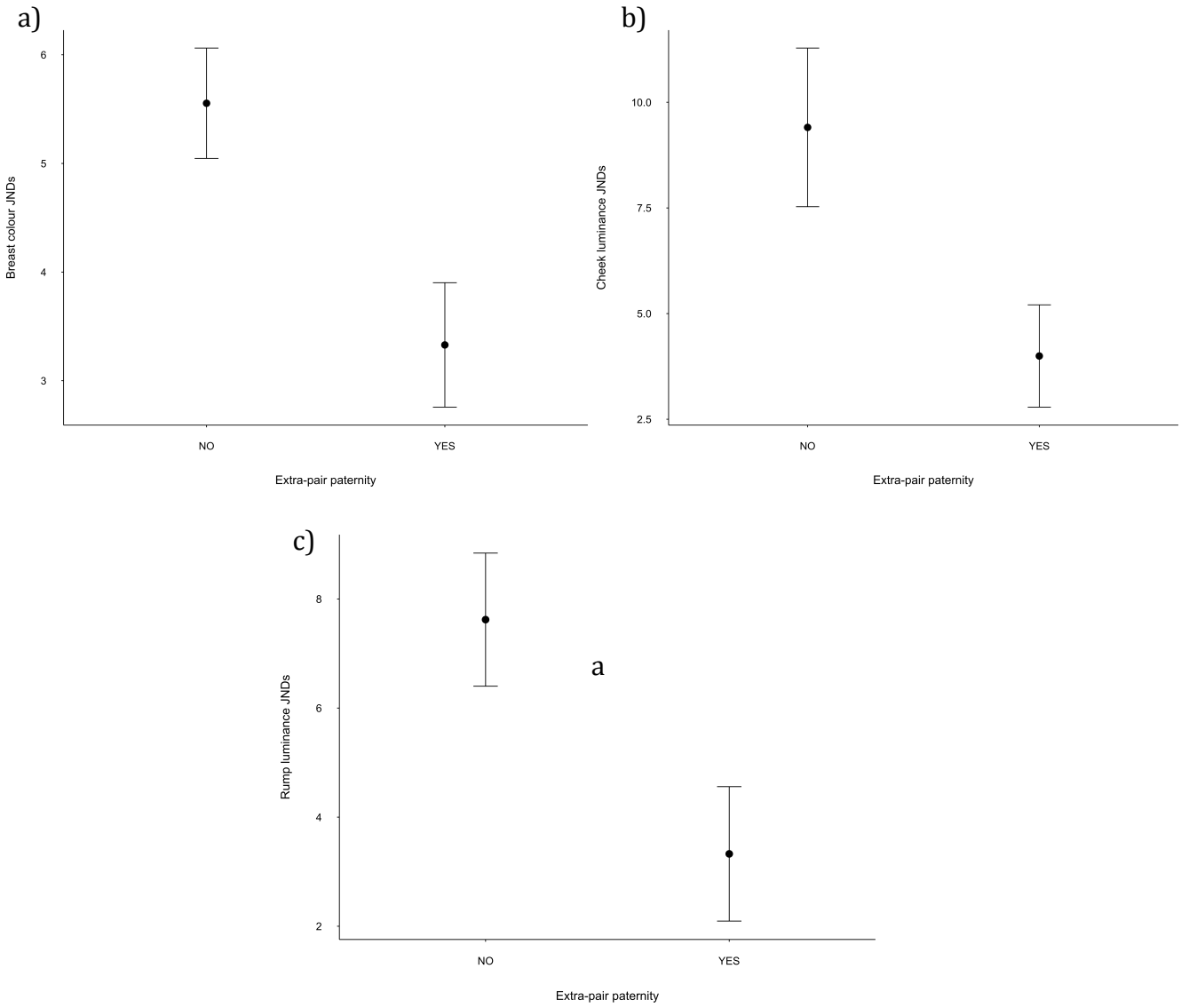

Figure 2. Colour change between seasons (2013-2014) expressed as JND scores and paternity gain (yes/no) (a) in the yellow breast colour, (b) in the white cheek luminance and (c) in the blue-green tail luminance.

DISCUSSION

In a previous study we found that the conditions experienced during a single reproductive event may have an effect on feather colouration obtained during the subsequent late summer moult, at least in the achromatic cheek (**Chapter 1**). Here, we further investigated the relationship between paternity, male quality during reproduction and colour change between seasons in several feather patches. As expected, during the spring of 2013, high quality males, as shown by colour and physiological variables, were more likely to sire extra-pair young. The following season these blue tit males showed similar chromatic colour in their yellow breast feathers and similar achromatic colour in their white cheek and blue-green tail feathers.

In the 2013 breeding season, we found that blue tit males with extra-pair nestlings outside the social nest had significantly more saturated carotenoid-based ornaments, which also showed higher hue. Yellow saturation is a good indicator of the amount of carotenoids deposited in feathers after they are obtained through diet (Saks et al. 2003; Senar et al. 2008). Thus, it is likely that these males were better foragers, as shown in another blue tit population located nearby (García-Navas et al. 2012). A similar relationship was found in great tits (*Parus major*), but in another colour variable. Pagani-Núñez and Senar (2014) suggested that males with higher values of hue in their carotenoid-based ornament displayed their ability to exploit alternative high quality prey (i.e. arachnids). As saturation, hue could be regarded as a measure of carotenoid content, but it could also reveal the presence of small amounts of melanin (Andersson and Prager, 2006). However, the relationship between melanin deposition on feather ornaments and condition has yielded contradictory results (see Hegyi et al. 2007 and references therein), and in the blue tit, the presence of melanin in yellow feathers is unstudied. Nonetheless, blue tit males with more ornamented yellow breasts were likely to be preferred by females for extra-pair matings because they were high-quality males. In fact, previous

studies in the same blue tit population have reported that these males harboured less blood parasites (del Cerro et al. 2010; Badás et al. 2017-**Chapter 3**).

Unexpectedly, in the spring of 2013, males with extra-pair nestlings were also more likely to harbour more blood parasites of the coccidian *Lankesterella valsaininesis*. Coccidial parasites can disrupt carotenoid absorption in the bird's intestine, and thus, these infections are expected to result in loss of feather colour in carotenoid-based ornaments (Brawner et al. 2000; Hõrak et al. 2004); but in this study, blue tit males that were more intensely infected by *Lankesterella* had more saturated yellow breasts. However, the parasites detected here were extra-intestinal stages of *Lankesterella* that infect lymphocytes in the blue tit's peripheral blood (Merino et al. 2006), so these coccidians are different from those found in other studies. Low parasite loads in infections by Lankesterellids may explain the lack of negative effects on the host's feather colouration and physiological variables, but further information on the parasite's virulence effect is lacking. An alternative, non-exclusive, hypothesis for this finding could be that males engaging in extra-pair copulations are more exposed to vectors that transmit *Lankesterella* parasites. The most common vectors for this blood parasite are mites and ticks (Lainson 1960), which require direct contact between individuals in order to infect new hosts. Individuals siring extra-pair offspring often engage in multiple extra-pair copulations with one or more females (Kempenaers et al. 1992), and thus, they are more likely to be in contact with infected individuals (Arnqvist and Kirkpatrick 2005).

Confirmation for this was found in our study: males that sired extra-pair young, also had higher haemoglobin concentration in blood, probably as a result of higher activity when searching for additional matings. Indeed, whole-blood haemoglobin levels have been related to adult performance, because an increase in haemoglobin is needed to circulate more oxygen throughout the body during demanding activities (Scott and Milsom 2006). Moreover, higher haemoglobin concentrations in blood may also indicate that these were

high quality individuals. Although in nestling blue tits, it has been recently shown that long-term variation in haemoglobin concentrations were correlated with caterpillar abundance and higher fledgling success (Glądalski et al. 2016), showing that nestlings in better condition and better nutrition status tended to have higher haemoglobin levels. To our knowledge, the present study is the first one showing a relationship between haemoglobin concentration in blood, paternity and feather colouration in adult blue tits.

Blue tit males may also gain extra-pair paternity when they are more colourful in other ornaments. Males that gained additional paternity were more saturated in their blue crown feathers. Delhey et al. (2007) found a similar relationship between crown hue and the siring of extra-pair offspring in three consecutive years; adult males with more UV-shifted crown hues were less likely to sire extra-pair young than less UV-shifted adults. However, the strength of this pattern varied among years and it was not consistently statistically significant (Parker 2012). In our study, we must be cautious when interpreting this result because the model only included colour variables, although it benefited from a bigger sample size.

Another noteworthy point is that, between seasons, males that sired extra-pair young and, overall, fathered more offspring, changed colour less. Lower JND scores indicated reduced changes in plumage colouration in several ornaments: the yellow breast, the white cheek and the blue-green tail. Therefore, males that were already more ornamented in several feather patches in the 2013 breeding season (more saturated in their yellow breast feathers and blue crown, and a tendency to have brighter blue-green tail feathers), retained high quality ornaments for the consecutive breeding event. This is in accordance with another study in blue tits, which suggested that the change in carotenoid-based colouration between seasons may depend on quality in both sexes (Doutrelant et al. 2012). In our study, the less pronounced changes in colouration may indicate that even after a costly reproductive event, some males can access the necessary

resources to preserve feather colouration and honest signalling. Indeed, increasing reproductive effort reveals a trade-off between the resources allocated to reproduction against those allocated to ornamentation in males of several bird species (Gustafsson et al. 1995; Griffith 2000; Siefferman and Hill 2007). High quality individuals may be able to cope with the increased costs of seeking extra-pair matings.

All polygynous males in this study were second years or older. The same relationship between older age, increased crown colouration and paternity in the blue tit has been reported before in other European populations (Delhey et al. 2006a; Vedder et al. 2011). However, the opposite was found in another population, with extra-pair paternity being almost absent in older males (Johannessen et al. 2005). In this study population, younger males were more likely to father extra-pair offspring in another breeding season (Badás et al. 2017-**Chapter 3**). These findings suggest that the link between male age and siring of extra-pair offspring varies among populations and breeding seasons. Future studies in the present blue tit population may confirm or disprove whether the pattern seen in this study population is maintained among several reproductive years.

In conclusion, our study highlights that gaining paternity might be beneficial for male blue tits not only during the breeding season, but also for the following reproductive event. High-quality individuals may opt for a strategy in which they benefit from the siring of extra-pair paternity without compromising feather colouration in several ornaments. The potential costs of infection by certain blood parasites such as extra-intestinal coccidians and their effects on feather colouration remain unclear. In the future, particular attention should be given to the risk of parasitic infections that may result from engaging in extra-pair copulations, and to the fact that high-quality individuals may overcome these costs.

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PART III

AGEING AND REPRODUCTION

Chapter 5

'Study the past if you would define the future.'

—Confucius

This chapter reproduces entirely the manuscript:

E.P. Badás, J. Martínez, J. Rivero-de Aguilar, F. Miranda, J. Figuerola and S. Merino (2015) Ageing and reproduction: antioxidant supplementation alleviates telomere loss in wild birds. *Journal of Evolutionary Biology* **28:** 896–905

Ageing and reproduction: antioxidant supplementation alleviates telomere loss in wild birds

E.P. BADÁS, J. MARTÍNEZ, J. RIVERO-DE AGUILAR, F. MIRANDA, J. FIGUEROLA, AND S. MERINO

Abstract Reproduction is inherently costly. Environmental stressors, such as infection and limited food resources, can compromise investment at each breeding attempt. For example, recent data on captive birds showed that increased reproductive effort accelerates ageing. However, the effects of nutritional status and infection on ageing remain unknown. Telomeres function as protective caps at the ends of eukaryotic chromosomes, and changes in telomere length is a commonly used proxy for ageing. To partially address the mechanisms of ageing following reproduction, we supplemented, medicated or administered a combined treatment to wild blue tits (*Cyanistes caeruleus*) breeding in central Spain during 2012. The nutritional supplement consisted of two different antioxidants, while the medication was an antimalarial treatment against blood parasites. We evaluated the effect of these manipulations on reproductive success and parasite loads in the first breeding season, and on changes in telomere length between two consecutive breeding seasons. Supplemented birds showed no reduction in blood parasite infections in 2012, although they exhibited higher body mass and fledging success. The antimalarial drugs reduced infections by several parasite species, but this had no effect on fitness parameters. In the following season, telomeres from supplemented birds had shortened less. Altogether, we found that supplementation with antioxidants provided fitness benefits in the short term and reduced telomere loss a year following treatment. Our results provide indirect empirical support for accelerated telomere loss as a cost of reproduction.

Keywords Blood parasites, *Cyanistes caeruleus*, fledging success, medication, nutritional status, supplement, telomere length

INTRODUCTION

Telomeres are short tandem repeats of nucleotide sequences at the ends of eukaryotic chromosomes that maintain DNA integrity. They act as 'mitotic clocks', shortening with each round of cell division due to the end replication problem (Watson, 1972). When telomeres reach a critically short length, the cell enters a degenerative process of senescence which is eventually followed by apoptosis (Blackburn, 1991). In fact, ageing was first linked to telomeres in the early 1990s (Harley *et al.*, 1990), and subsequent studies confirmed that, in addition to the process of cell division, other factors accelerate telomere shortening; for example, oxidative stress (von Zglinicki, 2002; Epel, 2004). Hence, there is increasing evidence that telomere loss is a good proxy for ageing *in vivo* (Haussmann *et al.*, 2003; Blasco, 2005; Bize *et al.*, 2009; Barrett *et al.*, 2013).

During bird reproduction, higher levels of oxidative stress can be reached through increased parental effort (Alonso-Alvarez *et al.*, 2004; Metcalfe and Alonso-Alvarez, 2010; Christe *et al.*, 2012). When reproductive investment exceeds what is sustainable for parents, the costs on longevity become apparent through accelerated ageing (Santos and Nakagawa, 2012). Thus, studies increasing brood size in a range of organisms have shown that costly reproductive events had a negative impact on telomere dynamics on adult (Reichert *et al.*, 2014) and early life (Nettle *et al.*, 2013; Boonekamp *et al.*, 2014; Herborn *et al.*, 2014). The trade off between investment on current and future reproduction has puzzled evolutionary ecologists for decades (Williams, 1966), and still is a current topic of intense investigation (Roff and Fairbairn, 2007; Creighton *et al.*, 2009; Cox *et al.*, 2010). Traditionally, brood-manipulation experiments have reflected the relationship between investment in reproduction and telomere loss; however, it is currently unknown whether factors alleviating reproductive costs affect telomere shortening in the wild.

Proper nutrition is fundamental to reproductive success: when food is limited in an unfavourable environment, reproduction may be suspended in favour of metabolic processes that ensure survival (Wade *et al.*, 1996). In fact, birds from lower quality territories exhibited higher oxidative stress (van de Crommenacker *et al.*, 2011). Dietary antioxidants, such as vitamin E and methionine, are important during reproduction because they defend against oxidative stress toxicity (Giraudeau *et al.*, 2013). Methionine, an essential amino acid, is also an efficient scavenger of free radicals (Levine *et al.*, 1999; Elias *et al.*, 2005). The positive effects of certain diets on telomere dynamics have been reported in humans (Marin *et al.*, 2012) and mice (Vera *et al.*, 2013), but the effects of supplementation on telomere erosion in the wild are unknown. Moreover, these micronutrients improve immune system functioning, which is essential during breeding (Soler *et al.*, 2003; Brommer, 2004).

Impaired immune function during reproduction may also increase parasitic infections (Møller *et al.*, 2003). Numerous studies have demonstrated that avian malaria-like parasites are widespread (Pérez-Tris *et al.*, 2005; Merino *et al.*, 2008; Szöllösi *et al.*, 2011), and relapses from chronic infections are common during the breeding season (Valkiūnas, 2005). In addition to this, infected individuals result in increased oxidative stress (Isaksson *et al.*, 2013). Malaria is associated with susceptibility to oxidative stress, especially during energetically demanding stages of reproduction such as provisioning (van de Crommenacker *et al.*, 2012). In humans, telomere length shortens with infection and chronic diseases (Ilmonen *et al.*, 2008), but the link between parasitism and ageing in the wild remains understudied. A recent study in a wild warbler population investigated the relationship between chronic malaria and telomere loss and found evidence of the long-term costs of infection on ageing (Asghar *et al.*, 2015). Hence, telomere erosion is likely related to infection status during reproduction in wild birds.

To explore the association between telomere loss, nutritional status and parasitism, we designed a two-fold experiment during two consecutive breeding seasons in the blue tit (*Cyanistes caeruleus*). During the first season, one group of birds was administered with a supplement consisting of vitamin E and methionine, another group was treated against blood parasites using antimalarial agents, a third group of birds was treated with both the supplement and medication and a final group of birds acted as control. With micronutrient supplementation, we expect to generally enhance nutritional status, immune function and protection against antioxidant damage rather than assess the effects of individual compounds (i.e. methionine and vitamin E). In the combined supplement and medication group, parasites are targeted directly with the medication and indirectly through an enhanced immune response and nutritional status with the supplement. Thus, we expect parasitaemia reduction in both treatment groups, with a more acute reduction in the combined treatment group. This experimental design allowed us to investigate whether medication, supplementation and the combination of both (i) reflect improved fitness parameters in the short term and (ii) are efficient in reducing parasite loads. We then evaluated the effect of the treatments on fitness parameters and telomere shortening one year after administration of the treatment. If reduced parasitaemia and an increased supply of antioxidants alleviate reproductive costs, we expect to observe less change in telomere length in all experimental groups compared to the control group.

METHODS

Sample collection

The study was carried out during the 2012 and 2013 breeding seasons in a Pyrenean oak (*Quercus pyrenaica*) forest in central Spain (Valsaín, Segovia, 40°53'N, 4°01'W, 1200 m.a.s.l.). Blue tits had access to a total of 300 wooden nestboxes, with an

average occupancy of 25% of nestboxes per year (Fargallo and Merino, 1999). The present population has been under study since 1994 (Fargallo and Merino, 1999). Each season nestboxes are monitored to determine the impact of infection on host reproduction. Given the high prevalence of blood parasites, treatments could be blindly assigned (del-Cerro *et al.*, 2010).

In 2012, adults were captured at the nestbox twice, when nestling age was three and thirteen days (hatching date=day 0). Birds were ringed, weighed to the nearest gram and aged according to plumage characteristics (Svensson, 1992). Wing (±0.5 mm; method III following Svensson, 1992) and tarsus (±0.1 mm) lengths were also recorded. Nestling-provisioning rates were measured on day 10 at a subset of nests, using uniquely identifiable transponders attached to colour rings on the adult's tarsus. An antenna, connected to a data logger (Trovan, EID Iberica, Madrid), recorded entrances to/exits from the nest between the hours of 6:30 a.m. and 12:00 p.m. On day 15, nestlings were ringed; nestling mass and tarsus length were measured and unhatched eggs counted. After the breeding season, nests were inspected to determine which nestlings had successfully fledged.

At each capture, we obtained a blood sample via the brachial vein. One drop of blood was stored on an FTA card (Whatman, UK), and another was smeared on a slide. Blood smears were immediately air-dried and fixed in ethanol (96%), then later stained with Giemsa. After sampling the blood, we administered treatments by subcutaneous injections into the belly (each bird received a single injection at each of the two sampling occasions, see Fig. 1). Both individuals from the pair received the same treatment. Sets of four nests that shared similar hatching date (±1 day) and clutch size (±1 egg) were assigned to one of the following treatments: antimalarial drug, vitamin-methionine supplement, a combination of both or control. The dosage of antimalarials was considered subcurative based on the dosage for malaria treatment in humans. The treatment

consisted of an injection of 0.1 mg of primaquine phosphate (Sigma, St Louis, MO, USA) and 0.125 mg of chloroquine phosphate (Sigma, St Louis, MO, USA) solubilized in 20% solutol HS 15 (Sigma, St Louis, MO, USA), which is an innocuous solvent used when conventional vehicles are inadequate (Stokes et al., 2013). The supplement, calculated for a mean body mass of 10 g for adult blue tits, consisted of 0.9 mg α-tocopherol, 0.09 mg of mixed tocopherols and 1 mg of methionine in 20% solutol solution. The third treatment consisted of both antimalarics and supplements (i.e. primaquine, chloroquine, tocopherols and methionine), solubilized in 20% solutol. The control treatment was a 20% solutol solution. In all treatments, a total volume of 0.05 millilitres was administered per injection.

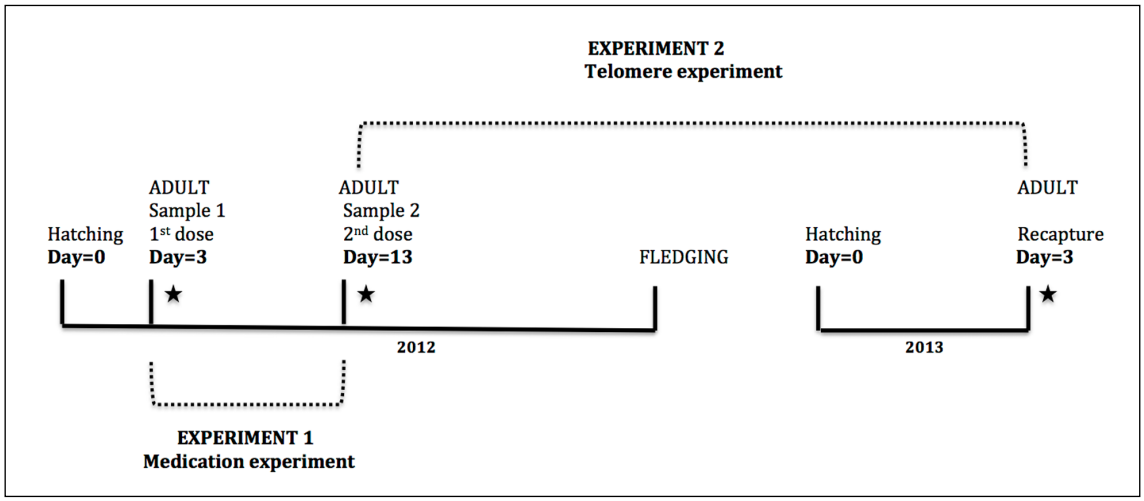

Figure 1. Schematic figure illustrating the experimental protocol during the two breeding seasons (2012 and 2013). Stages at which the adults were caught and administered with the treatments are shown (1st and 2nd dose). The star indicates that a blood sample was taken. The dashed line indicates which blood sample was used for each analysis (experiments 1 and 2).

Antioxidant dietary supplements administered orally do not result in increased concentrations of α-tocopherol in plasma compared with controls (Larcombe *et al.*, 2010). However, injectable lipid emulsions are commonly used as dietary supplements in veterinary practice (Driscoll, 2006), and studies with rabbits demonstrate the application of these emulsions as extravascular injectable vehicles for prolonged release of drugs (Wu

et al., 2014). The doses of antioxidants administered in the present study were based on the one used in previous studies (Soler *et al.*, 2003; de Ayala *et al.*, 2006). Rats supplemented for seven weeks with methionine showed oxidative damage of the liver when given excess methionine (Gomez *et al.*, 2009). Therefore, the dose/concentrations used are within the range of what is observed naturally in birds. Besides, based on evidence from Soler *et al.* (2003), these doses effectively reduced *Haemoproteus* parasite infections in magpies. Antimalarial treatments have previously been administered by subcutaneous injection in the same blue tit population, with significant effects on infection (Merino *et al.*, 2000; Martínez-de La Puente *et al.*, 2010), thus validating the use of this application method for all treatments in this study.

During the 2013 breeding season, no treatments were administered. Adults were captured and their blood sampled once (when nestlings were 3 days old, Fig. 1), following the protocol described above.

Parasitological and molecular analyses

For all samples, DNA was extracted from blood using a standard ammonium-acetate protocol and stored at -20ºC (Merino *et al.*, 2008). This DNA solution was then purified using silica filters to obtain a higher quality DNA (NZYGel pure, NZYtech, Lda. - Genes and Enzymes). DNA samples were quantified by spectrophotometry and adjusted to the same concentration (10ng/uL). For the 2012 blood samples (157 individuals from 79 pairs –one male was not captured), we detected and/or quantified the following parasites using two complementary methods (quantitative PCR (qPCR) and microscopic examination of blood smears): *Haemoproteus majoris* haplotype cyan2, *Plasmodium* sp. haplotype cyan1, and *Leucocytozoon* sp. haplotypes leuA, leuA1 and leuB. The variable *Leucocytozoon* A includes haplotypes A and A1 (see **General Methods Chapter**). In addition, a microscopic examination for the parasite *Lankesterella valsainensis* (Merino *et al.*, 2006) showed it was present at very low intensities (16% of samples were infected),

thus were quantified only by molecular methods (though qPCR results indicated that 36% of samples were infected). These quantifications were also included in the experiment, although the antimalarial treatment was not specifically targeting this parasite. We used relative qPCR with SYBR green (SYBR Selected Master Mix, Applied Biosystems) to amplify a fragment of the cytochrome B or 18S rRNA genes using a pair of species-specific primers for each parasite (Table 1 in the **General Methods Chapter**). Blood smears were also examined under high magnification (1,000x) to quantify the number of *H. majoris* juvenile/mature gametocytes. All blood samples were examined using an Olympus BX41 light microscope by EPB.

For telomere length analyses, we used samples collected during the second adult capture in 2012 and the only capture in 2013 (Fig. 1). Thus, the sampling universe for this experiment consisted of recaptured individuals only (68 individuals; annual return rate of 43.3%). After molecular screening for multiple parasite species (see above), DNA sufficient for telomere analyses was only available for 51 individuals. These samples represented a balanced proportion of recaptures from 2012 for each treatment group ($N_{control}$=14, $N_{supplemented}$=12, $N_{medicated}$=10, $N_{supplemented+medicated}$=15); the 2013 recaptures were not significantly skewed to the treatments administered in 2012 (GLM with binomial error distribution, c^2_3=213.83; P=0.802). We used the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as the control single copy gene. GAPDH primers were specific to the zebra finch but also amplify other bird species (Criscuolo *et al.*, 2009). The use of these primers in our samples was justified by checking non-variability of the control gene. The melting curves of the control gene cycles confirmed the lack of primer-dimer non-specific amplification, and the efficiency was close to 2 for all PCR plates (see the **General Methods Chapter** for further details). GAPDH was used as an internal control to normalize the amount of telomere sequence to the amount of DNA in the reaction. Telomere primers Tel1b and Tel2b were used at a concentration of 100nM; GAPDH-F/GAPDH-R primers were used at 200nM (Table 3 in the **General Methods Chapter**). The

final PCR volume was 20 µL containing 10 µL of Light Cycler 480 SYBR Green I Master (Roche) and 20 ng/µL of DNA. Telomere and GAPDH real time amplifications were performed on different plates, with each sample run in duplicate. RT-PCR-based methods for estimating telomere length are sensitive to the presence of intersticial telomeric sequences, and consequently are not adequate for estimating absolute telomere length (Nussey *et al.*, 2014). However, this is not a problem for our study because we are examining changes in the telomere length from individuals over time, and not absolute telomere length. Telomere PCR conditions were 10 min at 95°C followed by 30 cycles of 1 min at 56°C and 1 min at 95°C. GAPDH PCR conditions were 10 min at 95°C followed by 40 cycles of 1 min at 60°C and 1 min at 95°C. All PCRs were performed in a Light Cycler 480 RT-PCR System (Roche). Each 96-well plate included serial dilutions (40 ng, 10 ng, 2.5 ng, 0.66 ng of DNA per well) of DNA from a reference pool (the internal control) run in triplicate, which were used to generate the standard curves, and a blank control with no DNA. The slopes of the standard curves ranged from -3.649 to -3.460 with a R^2 value between 0.98 and 1.00; efficiencies ranged from 1.885 to 1.976 (further details in the **General Methods Chapter**). The coefficients of variation of the Cq values for the GAPDH and telomere amplifications were less than 5% in all samples following Criscuolo *et al* (2009). Sample level repeatability within and across plates was greater than 97.8% for GAPDH and telomere RT-PCR. Quantification cycle values (Ct) were transformed into normalized relative quantities (NRQs) using standard software (see Hellemans *et al.*, 2007 for formulas).

Statistical analyses

All analyses were performed in R v.2.14.0 (R Foundation for Statistical Computing, Vienna). First, we checked for pre-existing differences between experimental groups in 2012 (see the Appendix section). The full model was evaluated with respect to the significance of each explanatory variable. As treatments were sorted according to timing

of breeding and clutch size, medicated and control birds did not differ with respect to laying or hatching date, clutch size, or initial parasitaemia. Only medicated males showed higher infection intensity with *Leucocytozoon* B ($F_{3,65}$= 3.1941, p=0.029) than controls. The conditions prior to the experiment were accounted for by adding initial parasitaemia as a covariate in the subsequent analyses.

Next, we examined the effect of treatment on the intensity of the infection with linear or generalized linear models (GLMs), depending on the most appropriate error distribution. When there was evidence of overdispersion (Zuur *et al.*, 2009), likelihood ratio tests were used to compare the negative binomial and the analogous Poisson model, which confirmed that the negative binomial was more appropriate than the Poisson model. These analyses aimed to test the variation in parasitaemia with respect to the treatment; thus, individuals that remained uninfected during the course of the experiment (samples 1 and 2 from season 2012) were removed from the analyses. In any case, when the analyses included these individuals, the same conclusions were reached. All models included initial parasitaemia, sex, experimental group and the interaction between sex and experiment. By introducing initial intensity as a covariate in the analyses, we controlled for the pre-treatment differences (Merino *et al.*, 2000). The dependent variable (final parasite intensity) was log transformed when necessary, and residuals examined to check for compliance with each test's assumptions. The differences between treatment groups were evaluated using a t-test with Bonferroni correction or a sign test for related samples. When the assumptions of normality were violated and the variance between groups was highly different, we used the sign test to evaluate the difference in the median of parasitaemia between sampling occasions (Gibbons and Chakraborti, 1992). We also accounted for pre-treatment differences by matching repeated observations of the same subject. Full models were evaluated with respect to the significance of each explanatory variable. The effect size of the difference in parasitaemia for each treatment group was

computed as the Cliff's delta for nonparametric effect size estimates with the 'orddom' package in R (Rogmann, 2013).

In 2012, we also investigated the effect of treatment on host fitness. For the adults, changes in body mass were examined through an ANCOVA with initial body mass, tarsus length and sex as explanatory variables. Differences in female provisioning rates between treatments were tested using a Kruskal-Wallis test. Males were discarded from the analyses due to loss of transponders. For the analyses on reproductive success we used generalized linear models (GLMs) with binomial error for the proportion of hatched young that reached 15 days of age (fledging success). The effect of the adults' treatment on nestling body mass were checked using a linear mixed effect model in order to account for non-independence of brood mates (nest as random effect). Tarsus length and hatching date were included as covariates. In all cases, full models were evaluated with respect to the significance of each explanatory variable. After multiple testing on the same data, we used the false discovery rate (Benjamini and Yekutieli, 2001) to correct all p-values from the resulting models (see Table A1 in the Appendix for uncorrected p-values).

Finally, we explored the effect of treatment on telomere shortening. The variance is usually used as a measure of spread in ordinary least squares regression, but it is particularly sensitive to outliers, especially with low sample size. Two points in the combined supplemented and antimalarics group and one point in the vitamin only group appeared to have high leverage in our data set; excluding these data points generated further heteroskedastic problems. Therefore, we fitted an ordinal logistic regression to the complete dataset to control for skew and high leverage data points (further details on the statistical analyses in the Appendix). The rate of telomeric change was calculated as the difference in telomere lengths between 2012 and 2013, corrected for regression to the mean following the equation suggested by Verhulst *et al.* (2013). We used the change in telomere length as a dependent variable to control for pre-treatment differences in

telomere length (median test differences for the control vs. supplemented group, one sample sign-test: s=2 p-value=0.01) and treatment as explanatory variable. Sex or the interaction between treatment and sex were not included due to reduced sample size.

Fledgling success or nestling body mass in 2013 was not tested because nestlings were included in a different experimental design that year.

RESULTS

Efficacy of treatments in 2012

Supplementation had a positive effect on adult body mass. After correcting for initial body mass, sex and tarsus length, the ANCOVA revealed a significant effect of the treatment on final body mass ($F_{3,136}$=3.98, P<0.000, $N_{control}$=33, $N_{supplemented}$=41, $N_{medicated}$=35, $N_{supplemented+medicated}$=37). The natural reduction in body mass during the breeding season was mitigated only after supplementation with antioxidants (pairwise t-test with Bonferroni correction, control vs. supplemented group: P<0.000, and control vs. combined group: P=0.02, Fig. 2). This decrease was significantly different between the sexes ($F_{1,136}$=1.06, P=0.003, N_{female}=77, N_{male}=69), but not between treatment and sex ($F_{3,136}$=0.16, P=0.74, N=146). After correcting for tarsus length, nestling body mass in 2012 was not affected by the treatment of the adults ($F_{3,81}$=0.61, P=0.74, N=86 nests), nor were female provisioning rates (Kruskal-Wallis test: c^2_3=1.74, P=0.74). However, fledgling success was affected by the treatment (c^2_3=23.67, P=0.0001, N=74 nests), with the supplemented group having significantly more fledgling success compared to the control group (pairwise t-test with Bonferroni correction, control vs. supplemented, P=0.043, Fig. 3).

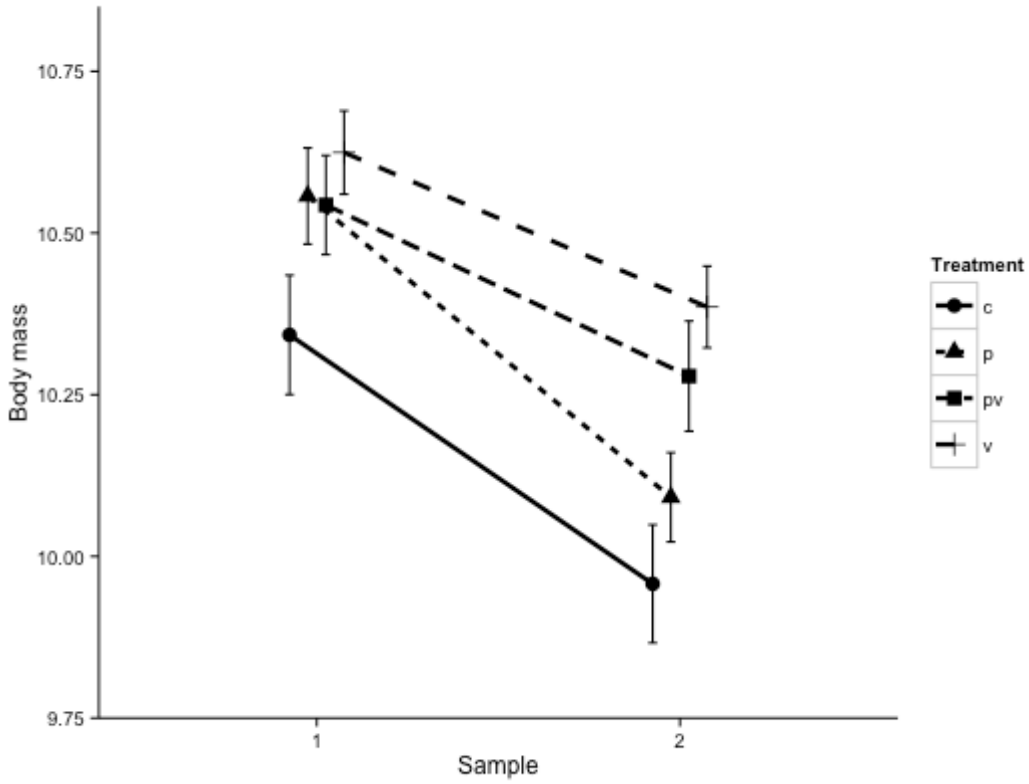

Figure 2. Change in adult body condition index with respect to treatment and sampling occasion. Bars denote standard error. Codes: control=c, antimalarial drugs=p, supplement=v, antimalarial and supplement=pv. Sample refers to initial sample (1=pretreatment) and final sample (2=post-treatment).

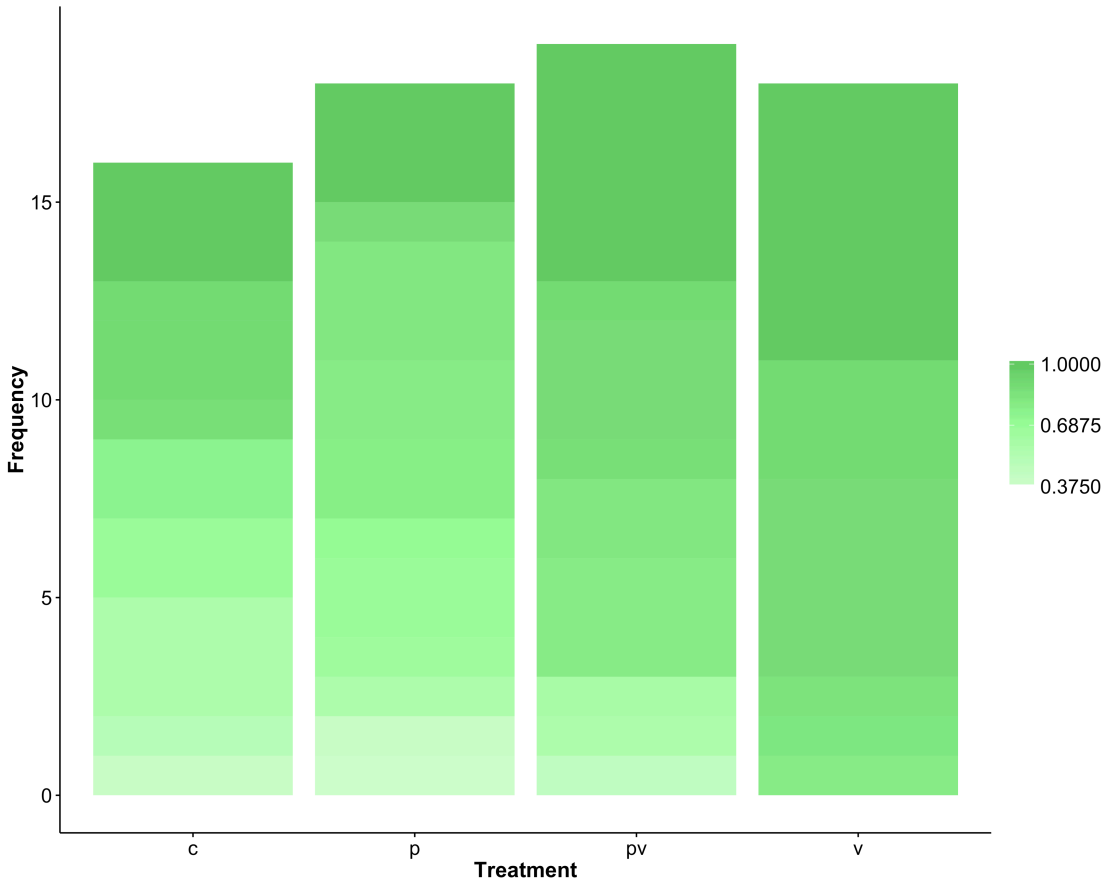

Figure 3. Fledging success. For representation purposes, we show the frequency of nests with its particular fledging success proportion. For each treatment group the frequency of nests represents a continuous of proportions ranging from 1 (all nestlings fledged) to 0 (no nestlings fledged). Codes: control=c, antimalarial drugs=p, supplement=v, antimalarial and supplement=pv.

Medication treatment was effective in reducing *Haemoproteus* parasitaemia when initial intensity was included as a covariate (negative binomial GLM, c^2_3=21.77, P=0.0002, $N_{control}$=26, $N_{supplemented}$=33, $N_{medicated}$=29, $N_{supplemented+medicated}$=29). Control birds naturally experienced a reduction in the number of mature parasites, however, a significant decrease was observed in birds administered with antimalarial drugs alone (sign test, s=8, P=0.012, effect size ES=-0.66) and in combination with antioxidants (sign test, s=9, P=0.031, effect size ES=-0.41). This treatment was also effective against *Leucocytozoon* B (negative binomial GLM, c^2_3=19.35, P=0.0006, $N_{control}$=27, $N_{supplemented}$=28, $N_{medicated}$=25, $N_{supplemented+medicated}$=29). In this case, parasitaemia by *Leucocytozoon* B was significantly reduced in birds receiving antimalarial drugs alone (sign test, s=6, P=0.0073, effect size

ES=-0.52) whereas control birds experienced an increase in infection intensity. Finally, *Lankesterella* infections were also significantly affected by medication (ANCOVA, $F_{3,57}$=8.42, P=0.0003, $N_{control}$=17, $N_{supplemented}$=17, $N_{medicated}$=19, $N_{supplemented+medicated}$=13) when antimalarics (pairwise t-test with Bonferroni correction, P=0.0238) and antimalarics with supplement (pairwise t-test with Bonferroni correction, P=0.0099) were administered. Infections by *Plasmodium* (ANCOVA, $F_{3,70}$=1.81, P=0.74, $N_{control}$=20, $N_{supplemented}$=24, $N_{medicated}$=14, $N_{supplement+medicated}$=21) and *Leucocytozoon* A (Poisson GLM, c^2_3=2.61, P=0.71, $N_{control}$=34, $N_{supplemented}$=36, $N_{medicated}$=33, $N_{supplemented+medicated}$=37) were unaffected by any treatment.

Change in telomere length between 2012 and 2013

All recaptured individuals from 2012 (n=54) had reduced telomere lengths in 2013, except for three individuals. Overall, telomere lengths were reduced by 16.44% in the control, 16.33% in the supplemented plus antimalarics, 11.56% in the antimalarics and 6.43% in the supplemented group. Administration of these treatments in 2012 had an effect on telomere shortening, based on the ordinal logistic regression (Likelihood ratio score vs. null model, c^2_3=12.62, P=0.01, N=51). Supplemented birds showed significantly less change in telomere length (Wald's Z=-3.14, P=0.002) (Fig. 4).

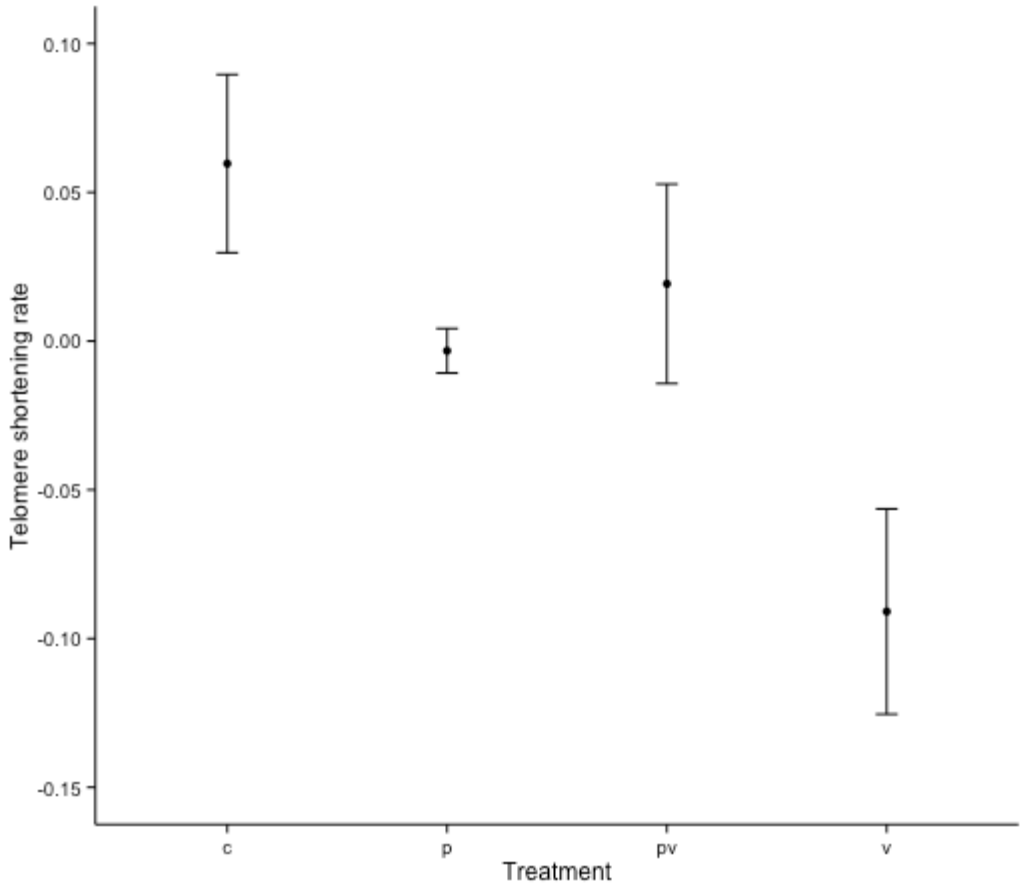

Figure 4. Telomere shortening rate with respect to treatment. The change in telomere length is corrected for regression to the mean using the equation suggested by Verhulst *et al.* (2013). This equation uses the corrected mean centering of the values from 2012 and 2013 measurements, thus, the rate is set to a central zero. Codes: control=c, antimalarial drugs=p, supplement=v, antimalarial and supplement=pv.

The effect of treatment in 2012 was not reflected in breeding parameters in 2013, such as clutch size (c^2_3=7.19; P=0.84), hatching date (c^2_3=13.69; P=0.75) or laying date (c^2_3=27.19; P=0.5).

DISCUSSION

Telomere shortening has recently been used as a biomarker for cellular aging processes in birds (Bize *et al.*, 2009; Barrett *et al.*, 2013). As hypothesized, antioxidant supplementation had positive effects on telomere dynamics, supporting the idea that nutritional status plays an important role in cellular functioning during reproduction. Recently, Reichert *et al.* (2014) showed that a single costly reproductive event shortened adult zebra finches' telomeres, which were not restored one year later. Our results in blue tits provide the first experimental evidence that antioxidants, such as vitamin E and methionine, decelerate telomere shortening *in vivo*. Supplementation alleviated the costs of reproduction, in such a way that telomere loss was significantly reduced one year after administration. For example, by providing extra reserves for methylation, methionine could help maintain DNA integrity and thus counteract telomere shortening (Ligi, 2011). The role of methionine in maintaining genome integrity has been suggested previously. In humans, low reserves of mediators in the DNA methylation pathway correlate with shorter telomeres (Benetti *et al.*, 2007; Fenech, 2012). Restriction of amino acids other than methionine have also been shown to prevent telomere shortening in rat livers (Tanrikulu-Kucuk and Ademoglu, 2012). In another study, a single intraperitoneal injection of a synthetic antioxidant modulated DNA methylation in rats (Vanyushin *et al.*, 1998). Supplementation with vitamin E could also explain the positive effects seen on telomere dynamics in this study. Dietary intakes of vitamin E were associated with longer telomeres in humans (Xu *et al.*, 2009), while *in vitro* experiments in human skin fibroblasts demonstrate that vitamin E restores telomerase activity and protects against telomere erosion (Makpol *et al.*, 2010). The reduction in telomere loss may be the result of an increased antioxidant capacity. Indeed, subcutaneous injection of the antioxidant melatonin increased antioxidant activity in rat liver (Manikonda and Jagota, 2012). To confirm this hypothesis in blue tits, future experimental work that also included

measuring circulating levels of antioxidants is needed. Surprisingly, the experimental group that received the combined treatment (primaquine, chloroquine, methionine and vitamin E) did not have a significant change in telomere shortening compared with the control birds. Primaquine is used to remove human malaria both at the tissue and blood stages of the parasite; chloroquine works mainly against the blood stage of the parasite (Baird and Rieckmann, 2003). The use of such drug associations for the treatment of human (Desjardins *et al.*, 1988) and avian malaria (Graczyk *et al.*, 1994) has been described before; but to date, there is no record of these compounds being used together with vitamin and methionine supplementation. The lack of an impact on telomere loss may be attributed to a negative interaction between the medication and antioxidants. Therefore, future experimental work in this blue tit population will focus on supplementation to better understand how telomere maintenance mechanisms may benefit from an enhanced nutritional status.

The benefits associated with supplementation also appeared during the 2012 breeding season. Birds suffered from decreased body mass during reproduction, but the supplemented birds benefited from a lower decline in body condition. Supplementation may have boosted the birds' performance by (i) reducing oxidative stress with the antioxidant properties of vitamin E and methionine (Alonso-Alvarez *et al.*, 2004), or (ii) by providing extra nutritional requirements. Indeed, we found that supplementation increased the probability that all nestlings in the brood fledged. This is consistent with previous findings using methionine supplementation in passerines (Soler *et al.*, 2003; Brommer, 2004). However, when we tested overall performance during reproduction (i.e. food provisioning rates), we did not find a significant effect of the treatment.

Methionine-fed magpie nestlings harboured less blood parasites (Soler *et al.*, 2003). Therefore, we hypothesized that supplementation would increase the immune response against parasites resulting in a decline in parasite load. However, in blue tits, no

significant effect on infection status was found after supplementation. To our knowledge, this is the first experiment that has combined vitamin E and methionine with an antimalarial treatment; therefore, other factors may explain the lack of an effect. For example, vitamin E may have provided nutritional and antioxidant properties to developing parasites (Müller, 2004), promoting growth. A similar pattern has been observed in studies where well fed parasitized hosts experienced higher mortality than underfed hosts (Pulkkinen and Ebert, 2004). In great tits (*Parus major*), female fleas that fed on food-supplemented hosts laid significantly more eggs (Tschirren *et al.*, 2007). In contrast, experimentally infected captive canaries did not experience higher parasitaemia after supplementation (Cornet *et al.*, 2014). Conflicting results regarding the effects of supplementation on parasitism highlight the need for further studies to better understand the ecology of host–parasite interactions. In any case, it is clear that, in blue tits, antioxidant supplementation improved fitness despite not having a significant effect on parasite load.

Faster telomere shortening rates have been related to stress (von Zglinicki, 2002) or diseases in humans and other mammals (Cawthon *et al.*, 2003). Contrary to expectations, the antimalaric treatment administered to adult blue tits had no effect on telomere shortening rates. The lack of an apparent relationship between parasitism and telomere loss may be explained by several factors. Firstly, blood parasites may have long-term detrimental effects on ageing processes that only become evident with time (Martínez-de La Puente *et al.*, 2010). Secondly, antimalaric drugs cause a range of side effects in humans. For example, combinations of primaquine and chloroquine over long periods of time can cause pruritus and anaemia (Kondrashin *et al.*, 2014). In birds, harmful side effects of such combination of drugs are unknown. In previous studies of this bird population, primaquine treatment successfully reduced malarial parasite infections and improved fitness (Merino *et al.*, 2000; Martínez-de La Puente *et al.*, 2010). However, during the 2012 breeding season, both primaquine and chloroquine were used, and a

different effect on blood parasite reduction was observed. Thus, the use of these drugs possibly masked a positive effect on telomere erosion in medicated blue tits.

Overall, we showed positive effects of antioxidant supplementation during the reproductive attempt and one year after. The short-term effects were seen in body mass and fledgling success, while the long-term effect was evident by a slower rate of telomere shortening. These findings constitute experimental evidence for linking nutritional status during a costly reproductive event and ageing. One of the costs of increased reproductive effort is an accelerated ageing rate as revealed by telomere erosion (Reichert *et al.*, 2014). In this study, without manipulating reproductive effort, we showed that improving nutritional status reduced telomere erosion one year after the breeding attempt. In the future, particular attention should be given to supplementation experiments and telomere maintenance mechanisms to understand how costs of reproduction affect ageing.

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APPENDIX

Pre-experimental conditions in 2012

In order to test the pre-experimental conditions we used the available data on all occupied nests in the populations (N=89 nests). Due to missing values and multiple DNA analyses (several PCRs were conducted on the sample samples), 79 nest pairs were used for the subsequent analyses. *Leucocytozoon* prevalences were not comparable between methods, since the qPCR was able to detect two haplotypes, indistinguishable by blood smear examination.

To look for differences in parasitaemia before the experiment, we used generalized linear models with Poisson or negative binomial error distribution and log link function for count data using glm.nb from the MASS package (Venables & Ripley, 2002). For the molecular data analogous linear models with logarithmic transformation were designed in order to meet model assumptions. Tweedie error distribution (Dunn, 2013) was assumed for zero-inflated continuous data. Full models were evaluated with respect to the significance of each explanatory variable. Significance levels were set to 0.05 and differences among groups were evaluated using pairwise t-test comparisons with Bonferroni correction.

Statistical note on telomere shortening analyses

When we fitted a linear regression model to evaluate the effect of the treatment on the rate of telomere shortening, clear non-normality problems were detected (Shapiro-Wilks test, W = 0.92, P= 0.0015, N=54) and log-transformation did not solve these problems. The variance did not differ among experimental treatment groups (Levene's test: $F_{3,50}$= 1.94, P=0.13). Further exploration of the data revealed three points that departed from the expected distribution. Indeed, the studentized residuals (R_1=2.80; R_2=2.51; R_3=3.81), Cook's distance (D_1=1.44; D_2=1.02; D_3=1.91) and leverage values (h_1=0.08; h_2=0.07; h_3=0.06) associated to these points confirmed that these were outliers. Some authors suggest that excessively influential points can be excluded from the analyses if there is a substantial change in the structure of the model after deletion (Crawley, 2005). This is not the case in our study, since excluding these points gave qualitatively similar results in the analogous linear model ($F_{3,47}$= 2.869, P=0.0463; Tukey HSD post-hoc comparisons, supplemented vs. control contrast with Bonferroni correction p-adjusted=0.034, see Table A1). Furthermore, heteroskedastic problems arise (Levene's

test: $F_{3,47}$= 3.71, P=0.02) if the outliers are deleted, probably due to the reduced sample size among treatment groups ($N_{control}$=14; $N_{antimalarics}$=10; $N_{antimalarics+supplement}$= 15; $N_{supplement}$=12). We believe that these incongruences could be masking the correct interpretation of a linear model without these data points. Besides, the nature of these influential data points is unknown (i.e. observational outliers, qPCR errors), and therefore excluding them from the analyses would not be appropriate. Instead, we used a non-parametric approach to deal with heteroskedastic data, skew and outliers, allowing for the use of the complete data set. Considering the reduced sample size and the presence of outliers, the ordinal logistic regression is the most parsimonious model we can fit to our data.

Table A1. Corrected and uncorrected p-values from multiple testing.

Dependent variable	Independent variable	Uncorrected p-value	Corrected p-value
Adult body mass	Treatment	8.917E-08	9.81E-07
Adult body mass	Sex	0.001287	0.003267
Adult body mass	Initial body mass	2.2E-16	3.63E-15
Adult body mass	Tarsus	0.698175	0.73755
Adult body mass	Treatment x Sex	0.660076	0.73755
Nestling body mass	Treatment	0.6077	0.73755
Nestling body mass	Tarsus	0.00001	4.71E-05
Nestling body mass	Hatching date	0.00001	4.71E-05
Female provisioning rate	Treatment	0.63	0.73755
Fledgling success	Treatment	0.00002929	0.000107983
Fledgling success	Hatching date	0.2155	0.414138267
Fledgling success	*Haemoproteus* adult parasites	0.6757	0.73755
***Haemoproteus* intensity**	Treatment	0.000073	0.0002409
***Haemoproteus* intensity**	Sex	0.2826	0.490831579
***Haemoproteus* intensity**	Initial *Haemoproteus* intensity	2E-16	3.63E-15
***Haemoproteus* intensity**	Treatment x Sex	0.4047	0.667755
***Lankesterella* intensity**	Treatment	0.0001005	0.0003015
***Lankesterella* intensity**	Sex	0.4946096	0.709657252
***Lankesterella* intensity**	Initial *Lankesterella* intensity	0.00002945	0.000107983
***Lankesterella* intensity**	Treatment x Sex	0.1491203	0.307560619
***Leucocytozoon B* intensity**	Treatment	0.0002315	0.000636625
***Leucocytozoon B* intensity**	Sex	0.5713153	0.73755
***Leucocytozoon B* intensity**	Initial *Leucocytozoon* B intensity	5.834E-07	3.85E-06
***Leucocytozoon B* intensity**	Treatment x Sex	0.2258936	0.414138267
***Leucocytozoon A* intensity**	Treatment	0.4561	0.709657252
***Leucocytozoon A* intensity**	Sex	0.6382	0.73755
***Leucocytozoon A* intensity**	Initial *Leucocytozoon* A intensity	5.834E-07	3.85E-06
***Leucocytozoon A* intensity**	Treatment x Sex	0.4834	0.709657252
***Plasmodium* intensity**	Treatment	0.5406	0.73755
***Plasmodium* intensity**	Sex	0.7152	0.73755
***Plasmodium* intensity**	Initial *Plasmodium* intensity	0.9015	0.9015
***Plasmodium* intensity**	Treatment x Sex	0.0237	0.05214
Telomere shortening rate	Treatment	0.0055	0.012964286

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INTEGRATIVE DISCUSSION

The general aim of the present Thesis was to explore several indicators of individual quality in a passerine bird, the blue tit (*Cyanistes caeruleus*). For birds, the assessment of quality in conspecifics is essential in mediating mate choice and life-history decisions, in order to maximize reproductive success without compromising self-maintenance or survival. For evolutionary biologists, exploring whether honest signals and other physiological parameters are good indicators of quality contributes to a better understanding of life-history trade-offs. The results presented here confirm that the studied parameters are good indicators of individual quality in the blue tit.

Because parasites can exert a variety of negative effects on the host (Merino et al. 2000; Knowles et al. 2010; Martínez-de la Puente et al. 2010; Martínez-de la Puente et al. 2011), and these are especially evident during reproduction due to relapses of the infection (Valkiūnas 2005), the way in which birds cope with parasitic infections may be a good indicator of individual quality. In fact, we found that nestlings (**Chapter 2**) and females (**Chapter 3**) in our study population were generally in better body condition (indicated by higher body mass) when they were less infected by the avian malaria-like parasite *Leucocytozoon majoris* haplotype A. Nestling blue tits were also heavier at fledgling when they were reared in nests with reduced ectoparasite loads, in accordance with previous studies (Richner et al. 1993; Heeb et al. 1998; Brommer et al. 2011). It seems clear that the combination of physical harassment, blood parasite infections and loss of blood produced by multiple bitings may affect birds in term of condition (Tomás et al. 2008; Martínez-de la Puente et al. 2009). Males in the present blue tit population, on the contrary, did not show reduced body mass with increasing intensity in parasitic infections, but their feather coloration in several ornaments was altered.

Prior studies have evidenced that ornamental colouration may signal resistance to parasitic infections to conspecifics, as suggested by Hamilton and Zuk (1982) (reviewed in Hill 2006). Thus, more ornamented individuals are expected to harbour less parasites.

Here, we showed that feather colouration in several structural ornaments was disturbed when individuals were infected during a period when the immune system is hampered (namely adult reproduction and development) (**Chapters 1 and 2**):

(I) The achromatic white cheek. When adult male blue tits were more parasitized by the malarial parasite *Plasmodium* spp. during the breeding season, they grew more saturated white cheek feathers after the post-reproductive moult (**Chapter 1**). More saturation in an achromatic ornament may signal that this is not a high-quality ornament, because white feathers should show low saturation values (saturation is the amount of colour when compared to white light). Therefore, colouration in the white cheek seems to be related to previous infections by blood parasites in our study population. In accordance with a study in the pied flycatcher (*Ficedula hipoleuca*), infected individuals may moult early overlapping this costly activity with reproduction (Morales et al. 2007), which in turn, may reduce the amount of resources destined to structural organization in achromatic ornaments, like the white patch in blue tits. This is in agreement with our results from **Chapter 3**, because in a different breeding season, we found that males had more saturated white cheeks when they harboured more *Haemoproteus majoris* parasites. The fact that two different parasite species (*Haemoproteus* in the spring of 2012 and *Plasmodium* in the spring of 2013) negatively affected white saturation in blue tits may be explained by changes in climatic conditions between years, which could cause seasonal troughs in vector abundance (Møller et al. 2013). For example, the presence of water bodies, precipitation or temperature may affect biting midges' (the most common vector for *Haemoproteus*) and mosquitoes' (the most common vector for *Plasmodium*) abundance differently (Elbers et al. 2015; Krama et al. 2015).

(II) The green base of the tail and blue crown. Parasites can also disturb colour formation in structural ornaments during development. As mentioned above, nestlings are also immunodepressed individuals; and we showed that nestling blue tits suffering from

nest ectoparasite infestations and infections by the blood parasite *Leucocytozoon majoris* haplotype A developed more saturated blue-green tails in the nest and less bright blue crowns after the post-juvenile moult (**Chapter 2**). Stressful conditions during early-life (i.e. nest infestation by parasites and blood parasitic infections) may disrupt structural colouration in a secondary sexual ornament in the blue tit, the blue crown. This, in turn, could have important consequences for the bird's first breeding attempt, because females prefer to mate with males showing brighter blue crowns (Hunt et al. 1998; Sheldon et al. 1999; Limbourg et al. 2004; Szigeti et al. 2007; Limbourg et al. 2012).

The relationship between carotenoid colouration and parasitic infections in our blue tit population was not so straightforward as it may seem with structural ornaments. In nestlings, we could not find an effect on carotenoid-based colouration after an experimental reduction of parasites (**Chapter 2**). In adult male blue tits, colour change in the yellow breast was not related to previous infections (**Chapter 1**), but it was related to current infection by *Haemoproteus majoris* in another breeding season (2012) (**Chapter 3**). However, since feather colour is deposited on feathers during the post-reproductive moult, the observed less saturated yellow colour may reflect the conditions experienced during the previous reproductive season, the spring of 2011 (as suggested in **Chapter 3,** and confirmed by our results in **Chapter 1**). The observed relationship between yellow saturation and *Haemoproteus* infection remained significant the following season (2012) because those individuals may have been poor quality individuals suffering from a relapse of the infection. Unfortunately this does not explain why we found apparently contradictory associations between carotenoid-based colouration and parasitic infections in other chapters. First, female blue tits in the 2012 season showed more saturated yellow feathers in spite of harbouring more *Leucocytozoon majoris* haplotype A parasites (**Chapter 3**). Second, the reduction of several ectoparasites and blood parasites during development did not affect yellow colouration in nestling blue tits (**Chapter 2**). Carotenoid-based colouration could be more dependent on access to carotenoids through

diet than it is to parasite disturbance. Thus, females showing increased yellow saturation in the spring of 2012 might signal good foraging abilities at the time of the moult (which took place after the 2011 breeding season). Still, because they mated with low quality males (poor foragers as seen by their lower yellow breast saturation, see García-Navas et al. 2012) in the breeding season of 2012, we observed negative effects on eggshell pigmentation and other female characteristics (body mass, clutch size or parasitic infections) (**Chapter 3**). Indeed, some authors have suggested that females prefer to mate with males with better territories (Galvan et al. 2009; Sirkiä and Laaksonen 2009).

A noteworthy point is that individuals in wild bird populations are usually infected by multiple parasite species. A study on blackbird (*Turdus merula*) bill colour and multiple parasite infections highlighted that certain combinations of parasites in the same host may influence indicators of individual quality differently (Biard et al. 2010). Carotenoid-based colouration could then act as an integrative signal of current status of multiple parasite infection, whereby females may choose more ornamented males because this signals reduced infections of a certain parasite species at a certain period of time. Our results from **Chapter 3** may be in line this idea, because in our blue tit population, males with more saturated yellow breasts were less intensely infected by *Haemoproteus majoris* but harboured more parasites of *Plasmodium* spp. Future correlational and experimental studies should thus investigate the expression of carotenoid signals as a function of multiple parasites in order to disentangle whether carotenoid-based colouration may reflect access to carotenoids and/or the history of parasitism. For example, our results in carotenoid-based colouration in nestling blue tits revealed that during development, ectoparasite harassment and blood parasite infections did not incur is costs in for carotenoid colouration (**Chapter 2**). Coccidial infections (which were not measured in this study) may have stronger effects on carotenoid deposition on feathers (McGraw and Hill 2000; Hõrak et al. 2004; Baeta et al. 2008; László et al. 2009).

Finally, ageing measured as telomere loss proved to be another important indicator of quality. Individuals in good nutritional status suffer less costs from reproductions and therefore the may be slowing ageing.

To sum up, high-quality individuals can bear the costs of being infected by several parasites during costly activities, while maintaining colouration in structural ornaments that may indicate good-quality and help them maximize reproductive success (even through increasing extra-pair paternity). High-quality individuals may thus be able to invest in nutritional status, allowing them to maximize reproductive success without costs on self-maintenance in terms of accelerated telomere loss.

For all of the above, females may be assessing the information received by multiple signals in order to evaluate the quality of the informer (i.e. structural colouration may inform on the history of parasitism and carotenoid colouration on foraging abilities or territory quality). What is more, even within one trait, multiple messages can be provided to conspecifics (Senar et al. 2008; Mahr et al. 2016). For example in the blue tit, it seems that a single structural ornament, the achromatic white cheek could provide different pieces of information through saturation and brightness, namely parasitic infections and body condition (measured through body mass corrected by size) (**Chapter 1**).

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CONCLUDING REMARKS

1. In our study species, the blue tit (*Cyanistes caeruleus*), the conditions experienced during the breeding season may have important implications for the following season. High-quality individuals allocate resources efficiently during reproduction to immune defence (by maintaining reduce parasite loads) and self-maintenance (by maintaining higher body mass); which allow them to develop brighter feathers in an achromatic feather patch at the post-reproductive moult. This, in turn, may result in increased reproductive success in the following reproductive event, because they were able to mate with brighter partners.

2. Early-life conditions may have important consequences for the bird's first breeding attempt. Nestling blue tits that were reared in an environment with reduced parasites and had higher body mass at fledgling may indicate these favourable conditions through colour expression in two structural ornaments, the blue crown and the blue-green tail. Potential partners in the following reproductive season may prefer to mate with these individuals because they grew brighter blue crowns and less saturated green tails.

3. Our results suggest that there is assortative mating in the blue tit. Poor-quality males, as shown by paler colour in their yellow breast feathers and intense infections by blood parasites, paired with females that were also in poorer quality, as shown by reduced clutch size, lower body mass and increased eggshell pigmentation.

4. Females that paired with lower quality males laid more pigmented eggs. Increased eggshell pigmentation could be a result of (i) a poor courtship feeding behaviour, (ii) female differential allocation precisely because they mated with males in poorer condition, or (iii) low-quality pairs breeding in low quality territories.

5. Male characteristics, namely age, extra-pair paternity, ornamentation and parasitic infections, are important determinants in eggshell pigmentation in blue tits, and hence, might influence embryo development..

6. In our study population, older high quality males (as indicated by higher body mass and more saturated yellow breast feathers) were more likely to sire extra-pair offspring. Our results suggest that the engagement in extra-pair copulations increased likelihood of coccidial infections by *Lankesterella*, probably due to more direct contacts with infected individuals when searching for additional matings. In spite of this, high-quality males seem to be able to overcome these costs and benefit from higher reproductive success, without compromising feather colouration obtained for the subsequent seasons.

7. The use of an antimalarial treatment during reproduction reduced infections by blood parasites in adult blue tits, but this had no effects on fitness parameters or ageing. Future studies should be cautious with possible side-effects from medication during the bird's costly reproductive event, or focus on exploring the effects from multiple parasitic infections in the long-term.

8. Our results in adult blue tits suggest that alleviating the costs of reproduction can provide fitness benefits both in the short and long term. This was evidenced by higher body mass and fledging success in one season and reduced telomere loss one year after a supplement treatment during reproduction. Higher-quality individuals, with increased antioxidant reserves, may be able to maximize reproductive success without suffering from accelerated ageing resulting from increased parental effort.

CONCLUSIONES

1. En nuestra especie de estudio, el herrerillo común (*Cyanistes caeruleus*), las condiciones experimentadas durante la temporada de cría podrían tener importantes consecuencias para la siguiente temporada reproductora. Los individuos de mayor calidad son capaces de repartir de forma más eficiente los recursos entre el sistema inmune (manteniendo las infecciones parasitarias en bajos niveles) y el mantenimiento del propio individuo (logrando no disminuir su peso corporal); lo cual les permite desarrollar un plumaje más brillante en un parche acromático durante la muda post-reproductiva. A su vez, esta característica podría dar como resultado un incremento del éxito reproductor en la siguiente estación reproductiva, ya que los machos más brillantes se emparejaron con hembras también más brillantes.

2. Las primeras etapas del desarrollo podrían tener importantes consecuencias en el primer intento reproductivo de las aves. Los polluelos de herrerillo que se desarrollaron en un ambiente con menos parásitos y que tuvieron mayor peso corporal al abandonar el nido podrían señalizar estas condiciones favorables por medio de la expresión de color en dos ornamentos estructurales, el azul de la corona y el azul-verdoso de la cola. Las parejas potenciales durante la siguiente estación reproductora podrían preferir emparejarse con estos individuos que desarrollaron un plumaje con coronas más brillantes y colas menos saturadas.

3. Nuestros resultados sugieren que hay un emparejamiento selectivo en el herrerillo común. Los machos de peor calidad, como demuestra su pálido color en el amarillo del pecho y su mayor intensidad de infección por parásitos, se emparejaron con hembras que también eran de peor calidad, como demuestra su reducido tamaño de puesta, menor peso corporal y mayor pigmentación en el huevo.

4. Las hembras que se emparejaron con machos de peor calidad pusieron huevos más pigmentados. El aumento de la pigmentación del huevo puede explicarse por (i) el pobre alimento ofrecido por el macho durante el cortejo, (ii) asignación diferencial de recursos por parte de la hembra, debida precisamente a la menor calidad del macho, o por (iii) el establecimiento de la pareja en un territorio de baja calidad.

5. Las características del macho, como por ejemplo, edad, paternidad extra-pareja, ornamentación o infecciones parasitarias, son importantes determinantes de la pigmentación del huevo en el herrerillo y, por tanto, podrían influenciar el desarrollo embrionario.

6. En nuestra población de estudio, machos más viejos y de mayor calidad (reflejada en su mayor peso corporal y mayor saturación en el amarillo del plumaje del pecho) tuvieron más polluelos extra-pareja. Nuestros resultados sugieren que las cópulas extra-pareja incrementan el riesgo de contraer infecciones por coccidios como *Lankesterella*, probablemente como consecuencia de un mayor número de contactos directos con individuos infectados durante la búsqueda de emparejamientos adicionales. A pesar de ello, los machos de mayor calidad parecen ser capaces de superar los costes de dicha estrategia y beneficiarse de un mayor éxito reproductivo, sin comprometer su coloración para el siguiente periodo reproductivo.

7. El uso de un tratamiento antimalárico durante la reproducción redujo las infecciones por parásitos sanguíneos en herrerillos adultos, pero esto no tuvo efectos en parámetros indicadores de éxito reproductivo o envejecimiento. Futuros estudios deberán ser cuidadosos con los posibles efectos adversos de la medicación administrada durante un evento reproductivo costoso, o centrarse en explorar los efectos a largo plazo causados por las infecciones por múltiples parásitos.

8. Nuestros resultados en adultos de herrerillo sugieren que el alivio de los costes de reproducción pueden tener efectos beneficiosos tanto a corto como a largo plazo. Así lo demuestra el hecho de que los individuos suplementados durante reproducción tuvieran mayor peso corporal y mayor número de volantones en un periodo de cría, así como menor acortamiento de telómeros un año después. Los individuos de mayor calidad, con más reservas antioxidantes, podrían ser capaces de maximizar su éxito reproductivo sin incurrir en un envejecimiento acelerado debido al incremento en el cuidado parental.

'One never notices what has been done;
one can only see what remains to be done'

—Maria Salomea Skłodowska-Curie